SUPEROVULATION AND EMBRYO COLLECTION IN WOOD BISON *(Bison bison athabascae)*: TOOLS TO PRODUCE DISEASE-FREE EMBRYOS

A thesis submitted to the College of Graduate Studies and Research

in partial fulfillment of the requirements for the

Degree of Doctor of Philosophy

in the Department of Veterinary Biomedical Sciences

University of Saskatchewan

Saskatoon

By

JESUS MANUEL PALOMINO CANO

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for the degree of Doctor of Philosophy from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work, or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Head of the Department of Veterinary Biomedical Sciences

Western College of Veterinary Medicine

52 Campus Drive

University of Saskatchewan

Saskatoon

SK, S7N 5B4

ABSTRACT

Reclamation of Canada's threatened wood bison (*Bison bison athabascae*) herd is complicated by cattle diseases. As part of an overall goal to conserve bison genetics, five studies were conducted to develop or adapting present reproductive technologies to produce disease-free in vivo-derived wood bison embryos. In Chapter 4, the efficacy of pLH and hCG for inducing ovulation and whether the effect was related to the size of the dominant follicle at the time of treatment was examined in wood bison during the anovulatory season . Ovulation rate with hCG (94%) was nearly two times greater than with pLH (54%), and bison with a growing follicle of ≥10 mm had a greater ovulatory response than those of 8-9 mm. In Chapter 5, the efficacy of pLH and hCG after superstimulation with single or two doses (48 hours apart) of FSH diluted in 0.5% hyaluronan was determined in wood bison. A greater superovulatory ovarian response was found in cows treated with hCG vs. LH during the anovulatory and ovulatory seasons (6.6 vs. 2.8 and 6.3 vs. 3.8 corpora lutea respectively). In addition, dividing the dose of FSH two resulted in greater superovulatory response in wood bison. However, the number of corpora lutea was still lower than expected as compared to cattle using the same two dose method of superovulation (15 corpora lutea; Tribulo et al., 2012). Therefore, in Chapter 6, the effect of the addition of a low dose of eCG at the end of the superstimulation protocol on ovarian response and embryo quality was examined. Although the number of ova/embryos recovered was higher in this study when compared with previous reports in wood bison, no effect of eCG on the number of corpora lutea and embryo quality was found. In Chapters 5 and 6, the effect of exogenous progesterone on embryo quality in wood bison during the anovulatory season was evaluated. We found that progesterone did not improve the number of freezable embryos in either study. In Chapter 7, the effect of lengthening of FSH treatment protocol on superovulatory response and embryo quality

during the ovulatory and anovulatory seasons was examined. There was no effect of lengthening the FSH treatment protocol on ovarian response and embryo quality during the anovulatory season. However, embryo quality and ovulation rate were increased by the lengthened treatment protocol during the ovulatory season. Additionally, more freezable embryos (Grades 1 and 2) were obtained during the ovulatory season (1.8 embryos) vs. the anovulatory season (0.3 embryos). Overall, results confirm that superovulation can be performed in wood bison throughout the year, but a higher number of freezable embryos were obtained during the ovulatory season. The final chapter (Chapter 8) focused in the production of disease-free embryos in wood bison. Following superovulation, *in vivo*-derived wood bison embryos were exposed *in vitro* to *Brucella abortus* biovar 1. After incubation, embryos were submitted to the 10-step washing procedures recommended by the IETS to remove the pathogen. When the washing medium contained antibiotics, 100% *Brucella*-free embryos were obtained. These findings validate the washing procedures for the production of *Brucella*-free embryos in wood bison.

ACKNOWLEDGEMENTS

First and foremost I want to thank my supervisor Dr. Gregg Adams. I appreciate his contributions of time and ideas to make my Ph.D. experience productive and stimulating. I am also thankful for the excellent example he has provided as a successful researcher. I thank him for encouraging my research and for allowing me to grow as a research scientist.

I would like to extend my grateful thanks to Drs Murray Woodbury, Reuben Mapletoft, and Rob McCorkell for their permanent and unconditional willingness to ease my doubts and their substantial suggestions during the conduct of my research and redaction of my thesis. I also want to thank Dr. George Seidel for accepting to be my external examiner and for his commentaries on the thesis. In addition, I want to thank to my committee chair, Dr. Karen Machin, and I would like to acknowledge Drs Muhammad Anzar and Gaby Mastromonaco for their help during the development of my research. It was my pleasure working with all of you.

I also thank the funding sources that made my PhD studies possible. I was honored to receive a VBMS Graduate Student Scholarship and a Dean Scholarship through my program.

I want to express my sincere gratitude to Brad McKell, Nick Hawkins, and Elsie Dawn Parsons, summer students who helped in working with the bison in the Native Hoofstock Centre. Also many thanks to Brad Blackmore, and the workers at Goodale Farms for taking care of the maintenance of the bison.

I am grateful to my friends Rodrigo, Kylie, Irfan, Denish, Carlos, Hayder, Fernanda, Alisha, Lauren, and Awang, for the wonderful times we shared throughout my PhD studies that made my time at the University of Saskatchewan enjoyable.

Finally, I want express my gratitude to the University of Saskatchewan and the Western College of Veterinary Medicine, for having and supporting me during all these years.

DEDICATION

I would like to dedicate this thesis to my wife Miriam, whose love, patience, understanding and support provided me the strength I needed to achieve my goals; and to my beloved son Ricardo, whose innocence and happiness taught me the beauty of life.

I also want to dedicate this work to my parents. Words cannot express how grateful I am to my mother, Luzmila and my father, Demetrio, for all of the sacrifices that you have made on my behalf and for always supporting me in all my pursuits.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AI	Artificial insemination
cc	Cubic centimeter
CL	Corpus luteum
COSEWIC	Committee on the Status of Endangered Wildlife in Canada
eCG	equine chorionic gonadotropin
EINP	Elk Island National Park
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
hCG	Human chorionic gonadotropin
i.m.	Intramuscular
IOI	Interovulatory interval
IWI	Interwave interval
LH	Luteinizing hormone
mg	Milligram
MHz	Megahertz
mL	Milliliter
mm	Millimeter
P4	Progesterone
PGF	Prostaglandin F2α
PRID	Progesterone-releasing intravaginal device
SEM	Standard error of the mean
USA	United States of America
vs.	Versus
WBNP	Wood Buffalo National Park
ZP	Zona Pellucida

CHAPTER 1. GENERAL INTRODUCTION

1.1 The wood bison

The wood bison (*Bison bison athabascae*) is the largest land mammal in North America (Roe, 1957; Geist, 1991). Males can weigh between 816 - 998 kg, while females are between 363 - 544 kg (Soper, 1964). The other subspecies of bison, the plains bison (*Bison bison bison*), usually weighs 10 - 15% less than the wood bison (Stephenson et al., 2001). While the subspecies of bison resemble each other, but there are some distinctive external features (van Zill de Jong *et al.,* 1995). Two main characteristics that differ between the subspecies are the position of the hump, and the delineation of the cape margins. In wood bison the highest point of the hump is in front of the shoulder and there is no cape demarcation. In contrast, the apex of the plains bison's hump is at or behind the shoulder and the cape is sharply delineated (van Zill de Jong *et al.,* 1995; Stephenson et al., 2001). Apart from the size and weight, there is no apparent sexual dimorphism in bison (Reynolds et al., 2003).

Historically, wood bison herds inhabited a vast region of North America which included Alaska, the Yukon Territory, northeastern British Columbia, northern Alberta, and northwestern Saskatchewan (Soper, 1941; van Zyll de Jong, 1986; Larter and Gates, 1990; Stephenson et al., 2001). In spite of this vast range wood bison herds prefer to roam in specific areas with only sporadic outward migrations in search of more abundant vegetation (Hartop et al., 2009). As a result, the wood bison was considered to be the non-migratory subspecies in North America (Roe, 1957). This characteristic has been attributed to their foraging behavior (Roe, 1957; Larter and Gates, 1991). During the winter season wood bison prefer wetlands-associated meadows, open savannah-like plains, and dry grasslands. This is in contrast to the summer season when

wood bison move to coniferous and mixed forest habitat (Larter and Gates, 1991). All of these habitats are abundant in the northwest areas of the continent (Reynolds *et al*., 2003; Strong and Gates, 2009).

In general, wild bison are gregarious animals and their groups are comprised of several females and offspring with a strong linear dominance hierarchy. The dominant animals are usually the strongest, heaviest, fastest, and apparently have the least amount of body hair during the breeding season (Vervaecke et al., 2005). Three different groups of bison can be seen: i) matriarchal groups composed of cows, calves, and yearlings; ii) bull groups composed of solitary bulls, and iii) breeding groups composed of a bull and several females (Reynolds et al., 2003). The size of the matriarchal and breeding groups vary from 11 to 20 animals (Fuller, 1960). Bull groups typically only have 3 to 4 individuals (McHugh, 1958).

1.2 Ecological importance of the bison in North America

Bison contribute to the preservation of the fragile ecosystem balance in the woodlands (Mitchell and Gates, 2002). They create new landscapes and routes through their grazing and wallowing activities. Invertebrates consume bison fecal droppings and small mammals and birds use shed wood bison wool to build nests. Moreover, they represent an important nutrient source for predators and scavengers (reviewed in Sanderson et al., 2007). Bison are also a keystone species facilitating the preservation and restoration of tall prairie grasses (Knapp et al., 1999), as herd grazing activities, promote the growth of new tall prairie grass.

First Nation people and North American bison share a very strong traditional relationship. Bison have been a valuable source of food, clothes, and housing since the aboriginal Native American community first inhabited North America (Berger and Cunningham, 1994; Haines,

1995; Garret, 2007). In spite of the majority of bison herds are now privately owned, wild northern bison herds exist on tribal lands used by First Nations communities and remain as an important source of food and fiber (Garrett, 2007). Future projects for the reclamation of threatened Canadian wood bison herds should be developed with the participation and collaboration of First Nations people.

1.3 Population dynamics and bottlenecks

The wood bison population in North America in the 1800's was estimated to be approximately 168,000 individuals (Soper, 1941). However according to recent studies, this number may have been an underestimate (Stephenson et al., 2001). Regardless, wood bison herds were distributed from Yukon in the north to northern Alberta (Canada) in the south (van Zill de Jong et al., 1995; Stephenson et al., 2001). The wild population of wood bison in these areas decreased from thousands of animals to just 250 wood bison by the beginning of the 1900's (Soper, 1941). Overhunting and growth in the fur trade played an important role in the near extinction of wood bison in North America, similar to the plight of plains bison (Gates et al., 1992). The Canadian government then increased wood bison conservation efforts, through banning of hunting and poaching activities, and also designating the Northwest Mounted Police (from 1894 to 1911) as stewards to protect remaining animals (Soper, 1941). This enabled the population to double in size, reaching 500 wood bison during the period described above (reviewed in Gates et al., 2001).

In 1922 Wood Buffalo National Park (WBNP) was established in northern Alberta and south of the Northwest Territories in order to preserve the last herds of wild wood bison residing in Canada (Environmental Assessment Panel, 1990; Gates et al., 2001). WBNP is the largest

national park in Canada (44,807 km^2 or 17,300 mi^2) and at the time it was founded, WBNP contained approximately 1,500 wood bison (Environmental Assessment Panel, 1990). However, controversial governmental decisions brought wood bison and plains bison together in the park (Soper, 1941; Environmental Assessment Panel, 1990) by thethe translocation of 6,673 plains bison from the Wainwright Buffalo Park to the WBNP during the period from 1925-1928. This resulted in hybridization between plains and wood bison and also the introduction of two cattle diseases: brucellosis and tuberculosis (Environmental Assessment Panel, 1990; Soper, 1941; Tessaro et al., 1990). By the end of the 1940s the population of bison had increased to 12,000 individuals (Environmental Assessment Panel, 1990). However, in 1946tuberculosis was detected and the first confirmed case of bovine brucellosis was detected in 1956 (Tessaro et al., 1990; Environmental Assessment Panel, 1990). By the beginning of the 1970's the population of wood bison in the WBNP had grown to approximately 16,000 individuals (Mitchell and Gates, 2002). However, since then the population of wood bison has declined and by the late 1990's there were only 2,300 individuals in the park (Joly and Messier, 2001; Mitchell and Gates, 2002), possibly due to increased prevalence of brucellosis and tuberculosis. Cattle diseases weaken infected wood bison, which makes them more susceptible to predation, lowers survival during extreme winter seasons, and can lead to reproductive failure via induced abortions (Joly and Messier, 2004a). Since the WBNP contains the largest herd of wood bison in North America and the most genetically diverse population of wood bison in Canada (Wilson and Strobeck, 1999), the recovery and conservation of wood bison in the WBNP has recently been gaining more importance for Canadians.

Three wood bison recovery projects were initiated between 1963 and 2005 (Gates et al., 2001). First, in 1963, 19 pure (non-hybrid) wood bison that tested negative to brucellosis and

tuberculosis were transported from Needle Lake, WBNP, to Fort Smith, Northwest Territories, to establish a disease-free herd in the area. However, after an anthrax outbreak, 4 bulls, 10 females, and 5 calves were relocated from Fort Smith to the Mackenzie Bison Sanctuary, Northwest Territories (Gates et al., 2001; Nishi et al., 2002), which would later become the largest disease-free wood bison herd in Canada (Larter et al., 2000). Second, in 1965, 47 wood bison were transported from a disease-free herd located near Nyarling River, WBNP, to Elk Island National Park (EINP), Alberta (Blyth, 1995). Unfortunately, brucellosis and tuberculosis were later detected in this herd in 1969, and consequently adults and calves were separated to prevent further spread of disease (Nishi et al., 2002). Following this strict management intervention of the infected herd in 1971 the wood bison population in the EINP was declared free of brucellosis and tuberculosis (Gates et al., 2001). Since then, the EINP population has provided founding stock for free-ranging herds, captive wild herds, and the bison farming industry (Gates et al., 2001; Nishi et al., 2002). Finally, in 1996 the Hook Lake project was developed to establish a disease-free pure wood bison herd from captured free-ranging animals in the Slave River Lowlands in the Northwest Territories (Nishi et al., 2002). Between 1996 and 1998 newborn calves from the area were captured, given prophylactic treatment against brucellosis and tuberculosis, and annually tested for disease (Gates et al., 2001; Nishi et al., 2002). In spite of being isolated almost at birth from potentially infected herds, in 2005 a bull from the Hook Lake project was found to be infected with tuberculosis during a routine post-slaughter inspection (Lutze-Wallace et al., 2006). The remainder of the herd was subsequently culled revealing 13 additional cases of tuberculosis (Himsworth et al., 2010). In summary, only wood bison genetics from the EINP are being disseminated in Canada. By establishing new populations from a very

small number of individuals, founder effects may lead to the loss of genetic diversity (McFarlane et al., 2006).

1.4 The importance of the genetic diversity

Genetic diversity is required for animal populations to evolve under circumstances such as environment change in order to survive (Reed and Frankham, 2003). Reduced genetic diversity can result from demographic bottlenecks, founder effects, inbreeding, and genetic drift (McFarlane et al., 2006; Gates et al., 2010). The loss of genetic diversity could limit the ability of a population to adapt to environmental change and may lead to inbreeding depression (Frankham, 2005; McFarlane et al., 2006). This depression will increase the likelihood of population extinction because with less genetic resistance individuals from a population may not be able to survive habitat loss, pollution, stochastic events, etc. (Frankham, 2005).

The wood bison population has experienced two major demographic bottlenecks. At the end of the last century in Canada, nearly 168,000 animals were reduced to 250 individuals (Soper, 1941) and between 1970 and the 1990s the population of wood bison in the WBNP had declined from 16,000 to 2,300 animals (Mitchell and Gates, 2002). When populations reach such small numbers, inbreeding and genetic drift (i.e., loss of alleles) leads to a decrease in genetic diversity (Frankham, 2005; McFarlane et al., 2006; Gates et al., 2010). Therefore, there is a need to save genetic material from the diseased wood bison herds in the WBNP as this population exhibits the greatest genetic diversity in Canada.

1.5 Brucellosis

1.5.1 Characteristics of the disease

Brucellosis is a disease caused by gram-negative, coccobacilli, facultative intracellular bacteria of the genus *Brucella* (Corbel, 1997). In cattle, the disease is associated with large economic losses due to its chronic infection (Bernues et al., 1997). Bovine brucellosis is caused by *Brucella abortus* which has the potential to infect cattle, sheep, goats, and humans (Neta et al., 2010). In North America, only biovars 1, 2, and 3 have been reported (Olsen and Tatum, 2010). Biovar 1 has been the most common strain of *Brucella abortus* found in the WBNP (Tessaro et al., 1990). The disease leads to abortion in cows, typically during the third trimester of pregnancy, uterine infections resulted from retained placenta, and female infertility. In males, *Brucella abortus* infection also leads to orchitis and epididymitis (Garry, 2008; Olsen and Tatum, 2010). Additionally, synovitis is present in infected animals or either sex. The bacteria may be shed in milk, uterine discharge, and aborted fetuses, all of which may transmit the disease to healthy animals through direct contact, or indirectly through ingestion of contaminated feed or water (Neta et al., 2010). Usually, milk production will decrease due to secondary effects of abortion such as uterine infections (Radostits et al., 2007). Additionally, infected animals tend to have longer intervals between births which may also affect the economy of cattle producers (Radostits et al., 2007).

Bovine brucellosis is also a zoonotic disease that causing serious debilitation and even death in humans (Young, 1995). People at particular risk are dairy farmers, slaughterhouse employees, veterinarians, and laboratory technicians (Bossi et al., 2004). Infected people display chronic intermittent fever (undulant fever), profuse sweating, anorexia, fatigue, weight loss, and depression (Young, 1995). Transmission of the bacteria usually occurs after ingestion, inhalation, and/or direct contact of the pathogen on mucous membranes or abrasions in the skin

(Bossi et al., 2004). Therefore, brucellosis has also been identified as a potential biological weapon for acts of terrorism (Pappas et al., 2004).

1.5.2 Brucellosis in wood bison

In 1985 Canada's domestic cattle herd was declared brucellosis free (Environmental Assessment Panel, 1990). Despite this, bison herds in and around the WBNP are still infected with bovine brucellosis (Tessaro, 1986). The fact that a few cases of brucellosis have been reported in cattle suggests that the cows may have contracted the disease from wildlife reservoirs. Similar events have been reported in other countries (Olsen, 2010). In Canada, and particularly in WBNP, wood bison represent the main reservoir of brucellosis (Tessaro, 1986; Olsen, 2010). The close interrelationship between disease-free herds (cattle and bison) and wildlife reservoirs (e.g., *Brucella*-infected bison) in and around the WBNP may have played an important role in brucellosis transmission. Therefore, collaborative efforts will be needed to address and resolve the problem of brucellosis in wood bison (Rhyan and Spraker, 2010).

The presence of bovine brucellosis in bison herds at the WBNP was confirmed between 1955 and 1956 after post mortem inspection of three slaughtered bison that came from the park (Corner and Connell, 1958). *Brucella* bacteria were likely carried and transmitted by plains bison to wood bison during the translocations of the 1920's (Environmental Assessment Panel, 1990). Currently the prevalence of brucellosis in bison in WBNP is estimated at 31% (Joly and Messier, 2004b), but has the potential to increase if no measures are taken to control the disease. That domestic cattle and bison herds live in close vicinity to WBNP increase the risk of spreading the disease to healthy animals (Joly and Messier, 2004b, Nishi et al., 2006; Tessaro et al., 1990).

1.5.3 Effect of brucellosis on wood bison population dynamics

Brucellosis may affect the dynamics of wood bison populations through adverse impacts on reproduction. Bovine brucellosis has been considered the main cause of wood bison abortions in the WBNP (Joly and Messier, 2001), particularly during the winter (Joly, 2001; Joly and Messier, 2005). In addition, newborn deaths have also been linked with brucellosis, presumably due to the inhibitory effects on milk production in their mothers (Davis, 1990). Fertility is also compromised in *Brucella*-infected cows (Joly and Messier, 2005).

In addition to reproductive pathologies, brucellosis may also lead to bison population declines by increasing predation risk (Joly and Messier, 2004a). As wolves are the main predators to bison (Reynolds et al., 2003), it was thought that increases in wolf numbers were driving the decline in WBNP wood bison (Carbyn et al., 1998).Healthy wood bison herds appear less susceptible to wolf predation (Joly and Messier, 2004a). However, wolves do not solely hunt bison. They also hunt moose and other small mammals making it unlikely that predation is the sole driver of bison population declines (Reynolds et al., 2003). It is possible that wood bison population dynamics in the WBNP may be negatively affected by the interaction between cattle disease and predators (Joly and Messier, 2001; Joly and Messier, 2004a).

Likewise, stochastic events such as severe winter weather will reduce the availability of forage for bison. In turn this will decrease their body condition and increase susceptibility to disease and wolf predation (Environmental Assessment Panel, 1990; Reynolds et al., 2003). Therefore, the combination of a hard winter (weight loss due to scarce foraging areas), cattle disease, and predators may increase mortality in WBNP wood bison (Joly and Messier, 2001).

1.6 Alternatives for reclamation of diseased wood bison

Disease control in the WBNP is necessary for two reasons. First, cattle diseases have been affecting the population dynamics of wood bison in the WBNP since the early 1970's (Mitchell and Gates, 2002). This diseased wood bison population has decreased from 16,000 to 2,300 individuals in 20 years (Joly and Messier, 2001; Mitchell and Gates, 2002). Interestingly, a combined effect between disease and predation was found to be involved in this population decline (Joly and Messier, 2004a). Secondly, there is a latent risk of spreading cattle diseases from infected populations to disease-free wood bison herds and domestic livestock in and around the WBNP (Tessaro et al., 1993; Gates et al., 2001). For these reasons, in 1988 the Environmental Assessment Panel recommended the eradication of the existing bison population in an around WBNP to protect domestic cattle, disease-free wood bison and people in the area, and to repopulate the area with healthy wood bison from disease-free areas or obtained through the use of genetic salvage operations (Environmental Assessment Panel, 1990). However, the idea of depopulation was not well received by the community and other alternatives were not taken into account (Shury et al., 2015). Alternatives involving modern diagnostic methods, vaccination, and reproductive technologies may help in the reclamation of wood bison in the WBNP.

1.6.1 Diagnostic methods for brucellosis

The diagnosis of brucellosis is mainly based on serological tests (Nielsen, 2002). *Brucella abortus* contains O-polysaccharide (OPS), a part of its lipopolysaccharide (LPS) molecule on surface which can be selectively detected in a test. (Bundle et al., 1987). The OPS was also found in *B. melitensis* and *B. suis* and therefore serological diagnosis of these three species of *Brucella* uses this cell antigen (Nielsen, 2002). Serological techniques for diagnosis include the

Rose Bengal Test (RBT), buffered antigen agglutination test (BAPT), milk ring test, rivanol precipitation, and mercaptoethanol (Poester et al., 2010). Only RBT, BAPT and the milk ring test are recommended by the World Organisation for Animal Health (OIE) for brucellosis screening in international trade (Nielsen, 2002). Other modern serological techniques include agar gel inmunodiffusion (AGID), complement fixation test (CFT), and enzyme-linked immunosorbent assay (ELISA). The AGID has the advantage of distinguishing vaccine antibodies versus field infection using polysaccharide B that is part of the OPS (Poester et al., 2010). The CFT is widely used for confirmation of brucellosis in spite of its complicated protocol (Nielsen, 2002). The ELISA protocol has the disadvantage of being unable to differentiate vaccinal antibodies and field infection, resulting in false positive results in vaccinated animals (Poester et al., 2010).

Fluorescence polarization analysis (FPA) is a recent technique demonstrated to have high specificity and sensitivity in the detection of brucellosis in cattle (Nielsen et al., 1996). This technique measures the excitation by plane polarized light of a fluorescent molecule. The rate of rotation of this molecule is inversely proportional to its size. When the OPS is labeled with a fluorescein, its size increases (Poester et al., 2010). If the sample is positive, the measurable rate of rotation of the labeled-OPS will be reduced and the unlabelled OPS will rotate faster (Nielsen et al., 1996). The FPA is a rapid and cost-effective technique, is able to differentiate antibodies from vaccination, and can be conducted in serum, milk, or the whole blood (Nielsen et al., 1996; Nielsen, 2002; Poester et al., 2010). Interestingly, the FPA has been used to detect brucellosis in wood and plains bison (Gall et al., 2000). Gall et al. suggested that the FPA is a more efficient and accessible test to diagnose brucellosis in bison compared to other methods. However, a later study showed that specificity of the FPA was very low compared to culture techniques using

field samples from the Yellowstone area (Schumaker et al., 2010). Therefore, further investigation is needed to determine the diagnostic accuracy and true specificity of the FPA to detect brucellosis in wood bison in the field.

1.6.2 Vaccination

Brucellosis occurs globally except in countries where the pathogen has been eradicated (Seleem et al., 2010). Eradication of brucellosis in domestic animals in Canada and the USA was achieved by testing and culling infected animals from the herd, and vaccinating the uninfected animals (Forbes, 1980; Ebel et al., 2008). The most common vaccines used to prevent brucellosis in cattle are *B. abortus* strain 19 (S19) and *B. abortus* strain RB51(Seleem et al., 2010). The S19 had been used globally in past years, but has been banned in Canada and the USA (Yang et al., 2013). The problem with S19 is that it maintains the O-LPS antigen that induces anti-LPS antibodiesso the tests are not able to differentiate antibodies of naturally infected from vaccinated animals (Olsen and Tatum, 2010; Yang et al., 2013). Additionally, S19 induces abortion when given in pregnant bovine females (Beckett and MacDiarmid, 1985) and bison (Davis et al., 1991). Strain RB51, unlike strain 19, does not interfere with serological diagnosis (Moriyon et al., 2004) and does not produce abortion in cattle (Cheville et al., 1993). More importantly, RB51 protected 100% of animals from abortion when the vaccine was prepared with a field strain of *Brucella abortus* (Lord et al., 1998). Therefore, RB51 is currently the most reliable vaccine used in cattle vaccination programs in the USA (Yang et al., 2013). DNA vaccines have been developed using subunits of *Brucella abortus* to avoid the use of live vaccines (i.e., RB51) and to prevent abortion in animals and humans (Yang et al., 2013).

However, a single shot of a DNA vaccine is less efficacious than live vaccines in protecting against brucellosismaking several booster injections necessary.

In bison, initial studies have shown that RB51, given in a similar dose as in cattle, induced placentitis and abortion in pregnant plains bison cows (Palmer et al., 1996). Additionally, it was found that a single dose of RB51 had little efficacy in adult bison (Davis and Elzer, 1999). In subsequent studies revaccination of bison with RB51 has been reported to safely booster pregnant cows and increase its protective effect (Olsen and Holland, 2003). However, RB51 may remain in fetal tissues following the booster injection. More recently, vaccination of bison calves was proven effective in protecting against systemic infections and preventing placentitis and abortion during their first pregnancy (Olsen et al., 2003). Additionally, ballistic or remote and hand delivery of RB51 were effective at inducing immunity in calves and adults ($1x10^{10}$ CFU of RB51) but ballistic delivery may need a higher dose in adult bison (Olsen et al., 2002). Conversely, DNA vaccination using vectors encoding *Brucella* proteins bp26 and the trigger factor (TF) have been used successfully in plains bison to induce a strong immune response with specific immunity against *Brucella abortus* (Clapps et al., 2011). Therefore, vaccination and revaccination of adults and calves with the RB51 or DNA vaccines may provide an effective tool to aid in brucellosis eradication in wood bison in the WBNP.

1.6.3 Reproductive technologies

The WBNP herd contains the majority of wood bison genetic diversity in the world (Wilson et al., 2005). Genetic diversity is critical for population survival (McFarlane et al., 2006). Because diseased bison may transmit *Brucella* to surrounding cattle and subsequently humans, widespread culling of the diseased herd in the WBNP has been suggested to minimize

this risk (Environmental Assessment Panel, 1990). This would result in a substantial loss of genetic diversity which may affect future survivability of wood bison. Reproductive technologies have been developed to preserve the genetic material of several wild species and domestic animals (Solti et al, 2000). The application of these technologies in diseased animals may become critical to the preservation of endangered species. The production of disease-free embryos and gametes from diseased animals would allow for the creation of a germplasm bio-bank to preserve the genetic material of endangered or threatened species (Wild, 1992; Holt et al., 1999). Artificial insemination, estrus synchronization, superovulation, *in vitro* embryo production, gamete and embryo cryopreservation, and cloning are some of the technologies that have been developed in wild animals with relative success (Loskutoff et al., 1995; Comizzoli et al., 2000; Solti et al., 2000). New technologies have also demonstrated that pathogens can be removed from gametes and embryos in livestock to prevent transmission of diseases to healthy animals (Bielanski, 2007; Givens et al., 2007). In this way, assisted reproductive technologies may be used in wood bison to preserve the genetic diversity from the WBNP and eradicate potential cattle diseases from the area. The use of these techniques in wood bison could be essential to repopulation efforts, but their effective application of depends upon a basic and detailed understanding of the normal reproductive biology of this species.

1.7 Reproductive features in bison and other species

The reproductive physiology of bison is not well-documented, as compared with the bovine model. As the reproductive cycle has been characterized in cattle, rapid utilization of assisted reproductive technologies has then been able to increase reproductive efficiency and genetic merit across the last several years (Gordon, 2004; Mapletoft et al., 2009). Fortunately, in recent

years, bison reproduction has been studied more extensively (Goodrowe et al., 2007; McCorkell et al., 2013a) and this newly-acquired information may assist in the development of reproductive techniques for wood bison.

1.7.1 Puberty and sexual maturation

Sexual maturation refers to the time when the individual has a greater likelihood of reproducing (Kinder et al., 1994), and after puberty the individual is capable of reproducing successfully (Moran et al., 1989). Factors that influence sexual maturity in the bovine species are nutrition, season, and breed (Rawlings et al., 2008). Differences in the reproductive organs between cattle and bison may be attributed to the size of adult males and females (Haigh et al., 2000). Full development of these organs allows for performance of reproductive functions such as follicular development, ovulation, spermatogenesis, semen production, pregnancy, and parturition (Kinder et al., 1994).

The age of puberty in a female wood bison may be as early as 12 months (Green and Rothstein, 1991). However, the age of first calving will vary depending on extrinsic factors and may be approximately at 2 to 4 years (Reynolds et al., 2003). For example, only 5% of 2-year-old females give birth in the WBNP (Fuller, 1966), whereas ~12% of 2-year-old females were mothers at Wichita Mountain Wildlife Refuge (WMWR), Oklahoma, USA, (Shaw and Carter, 1989). In contrast, more than 52% of bison at WBNP and 73% at WMWR have offspring at the age of 3 years old (Fuller, 1966; Shaw and Carter, 1989). Therefore, the majority of bison cows reach sexual maturity and become pregnant at 2 years old. Nevertheless, age of conception will determine the long-term reproductive success in cows and post-pubertal growth (Green and

Rothstein, 1991). For example, early maturing females have a longer reproductive life than those females that mature later.

In bison bulls, age has been determined as the main factor for the onset of puberty (Helbig et al., 2007). Bison bulls reach puberty at 16 months of age in the wild. However, a bison bull that will be used for breeding should pass its soundness evaluation at approximately 24 months of age (Helbig et al., 2007).

1.7.2 Regulation of the ovarian function

The neuroendocrine system is composed of reproductive hormones and their secretory organs, such as the hypothalamus, anterior pituitary gland, ovaries, and uterus (Yen, 1999). The neuroendocrine system is responsible for regulating the majority of reproductive functions in mammals (Amstalden and Williams, 2015). Hormones such as gonadotropin releasing hormone (GnRH), luteinizing hormone (LH), follicle stimulating hormone (FSH), estrogens, progesterone, and prostaglandin F2 α (PGF) are involved in the regulation of the ovarian function and ultimately of reproduction (Yen, 1999). The period of recurring physiological changes controlled by reproductive hormones in females is called the estrous cycle (Amstalden and Williams, 2015).

The estrous cycle can be divided in two main phases: the follicular and luteal phase (Senger, 2003). During the follicular phase, the dominant follicle produces high levels of estradiol-17β that will be responsible for the external symptoms of estrus and will induce the preovulatory surge of LH at the end of the luteal phase (Stevenson, 2007; Amstalden and Williams, 2015). During the luteal phase the corpus luteum produces progesterone which will exert a negative feedback on levels of LH (Adams and Singh, 2015). Additionally, a continuous development of anovulatory ovarian follicular waves occur during the luteal phase. In each estrus cycle, two to

three follicular waves can be observed in cattle (Savio et al., 1988; Adams and Pierson, 1995). Each follicular wave is preceded by a surge of FSH (Adams et al., 1992b). Estradiol and inhibin, secreted by the growing follicles, suppress the secretion of FSH (Ginther et al., 1996; Adams, 1999). After that, LH is used by the dominant follicle as gonadotropin stimulant for growing (Ginther et al., 1996; Ginther et al., 2001b). A preovulatory surge of LH occurs after luteolysis, and ovulation is triggered (Edqvist, 1993). Ovulation occurs at 19 - 20 days and 22-23 days in a two- vs. three- follicular wave cycle (Adams et al., 2008). After ovulation, the ruptured follicle becomes the CL and secretes progesterone. If the animal does not become pregnant, the CL regresses (luteolysis), the production of progesterone stops, and a new cycle will begin (Pineda, 2003; O'Malley and Strott, 1999). Prostaglandin (PGF), which is secreted in the endometrial glands, is responsible for the regression of the CL (Pineda, 2003). The process of luteolysis is initiated by the secretion of oxytocin from the CL and its release into the endometrium. Oxytocin then binds to its receptors to stimulate the production and secretion of PGF in the endometrium. In ruminants, PGF arrives to the ovary mainly via local utero-ovarian through a veno-arterial mechanism (McCracken et al., 1999). If pregnancy occurs, the embryo trophoblast secretes recognition signals (e.g., interferon tau) which interrupt the production of PGF and its luteolytic effects on the CL (Bazer et al., 1998).

The secretion of GnRH into the hypothalamus and its subsequent transport to the anterior hypophysis through the adenohypophyseal portal circulation has been considered the primary endocrinological mechanism regulating female mammalian reproduction (Edqvist, 1993; Amstalden and Williams, 2015). GnRH is released in a pulsatile manner and binds to its GnRH receptors in the anterior pituitary to activate the synthesis and the release of the gonadotropin hormones, LH and FSH (Yen, 1999). The pattern of secretion of LH and FSH will depend of the

stage of the reproductive cycle of the female and its mechanism of regulation (Levine et al., 1995; Fink, 2000). For example, during the early luteal phase (low progesterone) the LH will be secreted with high frequency and low amplitude. During the mid-luteal phase (high progesterone) LH will be produced with low frequency and high amplitude. Finally, during the late luteal phase the pulse frequency will increase, until the preovulatory surge of LH occurs during the follicular phase (Amstalden and Williams, 2015). The two gonadotropins reach the ovaries through the blood circulation to govern and control ovarian activity. FSH stimulates the growth of ovarian follicles while LH is responsible of follicular growth, oocyte maturation, ovulation of the preovulatory follicle, and corpus luteum (CL) formation (Amstalden and Williams, 2015). The growing follicle and CL will produce estradiol-17β and progesterone, respectively (Yen, 1999; Cheng and Leung, 2000). These steroid hormones are synthesized in the ovary under the synergistic action of both FSH and LH and exert feedback actions (positive or negative) at the central (hypothalamus) and adenohypophyseal levels to control the release of GnRH and gonadotropins, respectively (Amstalden and Williams, 2015).

Estradiol-17β and progesterone are the two most important gonadal steroids produced in the ovary (Amstalden and Williams, 2015). In the ovarian follicle's granulosa cells, aromatase enzyme transforms androstenedione and testosterone to estrone and estradiol-17β, respectively (Jamnongjit and Hammes, 2006; Andersen and Ezcurra, 2014). The production of these hormones is regulated by the action of the pituitary gonadotropins (Yen, 1999; Fink, 2000). FSH and LH will induce the activation of aromatase in granulosa and theca cells to produce estrogens and LH will induce corpus luteum formation which ultimately will produce progesterone (Adams and Singh, 2015). The physiological action of estradiol-17β is to maintain the normal morphological and functional status of female reproductive organs and induce the preovulatory

surge of LH (Stevenson, 2007). Progesterone is produced mainly by the corpus luteum under LH stimulation (Pineda, 2003; O'Malley and Strott, 1999), and is responsible for the establishment and maintenance of pregnancy and the control of LH secretion (Stevenson, 2007).

The hypothalamic-pituitary-ovarian axis provides neuroendocrine control over reproduction (Amstalden and Williams, 2015). The axis controls ovarian activity through the release of GnRH and gonadotropic hormones that direct the functional status of the ovaries. Meanwhile, estradiol-17β exerts negative feedback on the pituitary for releasing FSH and progesterone exerts a similar feedback on LH secretion (Yen, 1999; Stevenson, 2007). On the contrary, estradiol-17β exerts positive feedback on the pituitary to increase the pulsatile secretion of LH (Speight et al., 1981). After luteolysis, a preovulatory surge of LH will occur and ovulation may be triggered. Therefore, high levels of estradiol-17β at the end of the luteal phase will ultimately elicit ovulation by inducing the preovulatory surge of LH (Edqvist, 1993).

Although reproductive patterns in bison resemble those of cattle, studies of neuroendocrine control over reproduction have not yet been conducted in bison (Goodrowe et al., 2007). We can speculate that the neuroendocrine mechanism of control of ovarian function is similar in both species (bison and cattle). However, bison are seasonal breeders, and thus, the neuroendocrine mechanism as known in cattle may occur only during the breeding season. In other seasonal breeders (e.g., horses and sheep), the mechanism of neuroendocrine control of reproduction described above occurs during the breeding season, but differs during the anovulatory (non breeding) season (Thiery et al., 2002; Donadeu and Watson, 2007).

1.7.3 Seasonality

The most notable seasonal changes in environmental conditions include: photoperiod, temperature, rainfall, and food availability (Karsch et al., 1984, Gündoğan et al., 2003). These extrinsic factors influence the reproductive process such that the calving season ultimately occurs when offspring have the greatest change of survival (Thiery et al., 2002; Malpaux, 2000). In short-day breeders, like bison, mating behavior occurs in the fall season when daylight is short (Thiery et al., 2002). This allows the calving period to occur during a favorable time of the year (spring) after 9 months of pregnancy. Conversely, in horses, due to a longer gestation length (11 months), mating occurs in late spring (long-day breeder) and, consequently, the foaling period occurs in early spring (Donadeu and Watson, 2007). Therefore, photoperiod (the duration of sunlight) seems to be the main factor controlling seasonality in these species (Malpaux, 2000).

In general, seasonal breeders use photoperiod to synchronize their endogenous biological rhythms (Lincoln and Short, 1980). Therefore, ovulatory and anovulatory periods can be observed each year (Thiery et al., 2002)**.** The photoperiod in seasonal species will determine their reproductive activity according to their nearness to the Equator (Malpaux, 2000). In areas that are far from the Equator, the days may have around 18 hours of light in summer whereas the hours of light may consist of around 8 hours during winter. In a seasonal species, the time of daylight exposure will determine the onset of the breeding season (Lincoln and short, 1980). In short-day breeders (i.e., bison), the breeding season begins in fall when light levels start to decrease and increase the secretion of melatonin from the pineal gland (Dahl et al., 2000). Melatonin is the primary messenger for the secretion and release of GnRH in seasonal species (Hazlerigg et al., 2001; Malpaux et al., 2001). During the breeding season, the hypothalamic-pituitary-ovarian axis functions similar to non-seasonal breeders. However, during the non-breeding season, estradiol-17β will prevent ovulation (Thiery and Malpaux, 2003).

During the anovulatory season, estradiol-17β behaves as a potent inhibitor of GnRH and LH secretion, exerting its action specifically in the anterior pituitary (Karsch et al., 1993; Thiery and Malpaux, 2003). Therefore, ovulation will not occur even if a dominant follicle is present. However, the mechanism used for the estradiol to inhibit LH is not well studied. One possibility is that estradiol binds to α receptors in the hypothalamus, stimulating the secretion of RFamide-related peptide which is a potent inhibitor of the GnRH secretion (Kriegsfeld et al., 2010). However, it was found that estradiol exerts a direct negative feedback on LH secretion in ewes (Arreguin-Arevalo et al., 2007). Estradiol also induces the secretion and release of dopamine in the hypothalamus, another inhibitor of the GnRH secretion (Anderson et al., 2001; Singh et al., 2009). Regardless, estradiol is the main hormone that prevents estrus and ovulation during the non breeding season (Karsch et al., 1993; Thiery and Malpaux, 2003).

1.8 Reproductive physiology in the female bison

Bison are considered seasonally polyestrous (Reynolds et al., 2003; Goodrowe et al., 2007). The breeding season starts in the early fall (August-September) and is characterized by follicular and luteal activity. The ovulatory season ends in early spring (March-April) and is followed by a period of seasonal anestrus which is interrupted just prior to the next breeding season (Rutley and Rajamahendran, 1995). During the anovulatory season, follicular activity is present but luteal activity is not. Therefore, ovarian function differs between seasons in different aspects that will be discussed as follows.

1.8.1 Ovarian function during the ovulatory season

Recently, the ovulatory season in wood bison was found to begin in late August at 52º North latitude (McCorkell et al., unpublished data). Although at 50º North latitude, the onset of the

ovulatory season occurs in late July or early August (Vervaecke and Schwarzenberger, 2006). After the first ovulation, the corpus luteum (CL) experiences a short lifespan (approximately 4 days), resulting in a short interovulatory interval (8 days [McCorkell et al., unpublished data]; 10 days [Rutley and Rajamahendran, 1995]). This short period may be sufficient to elevate the circulating levels of progesterone to reinitiate the normal estrous cycle in female bison. A similar pattern of short lifespan of the CL after the first ovulation was observed in wapiti (McCorkell et al., 2007) and in post-parturition cows (Murphy et al., 1990).

Ovulation and corpus luteum formation are physiological events that will occur throughout the ovulatory season (Goodrowe et al., 2007). Early studies in bison characterized the length of the estrous cycle (between 21 - 23 days) using hormonal measurements in urine and feces (Matsuda et al., 1996; Kirkpatrick et al., 1991). Preliminary work in our lab, using daily examination of ovaries by ultrasonography, revealed a pattern of two follicular waves during an estrous cycle of 20 days (McCorkell et al., unpublished data). The ovulatory size of the dominant follicle in the second wave was 14 mm in diameter, with the maximum diameter of the CL reaching 18 mm. A pattern of two-follicular wave occur throughout the ovulatory season and discontinued only by the occurrence of ovulation (McCorkell et al., unpublished data; Kirkpatrick et al., 1991). The last ovulation in the cycling female bison is observed in middle of April (Rutley and Rajamahendran, 1995), which coincides with the onset of the anovulatory season in bison. The period of transition from the ovulatory season to the anovulatory season has not yet been described in bison. However, we can speculate that this process occurs as described in other seasonal breeders such as sheep (Karsch et al., 1993; Thiery and Malpaux, 2003) and wapiti (McCorkell et al., 2007).

1.8.2 Ovarian function during the anovulatory season

While transition from the ovulatory season to the anovulatory season has not yet been described in bison, it has been studied in sheep (Rawlings et al., 1977). By the time of the last cycle of the ovulatory season in the ewe, there is insufficient rise of LH surge and consequently no ovulation will occur (Rosa and Bryant, 2003). Thyroid hormones may be implicated to prevent the LH surge and, thus, might be responsible for the beginning of seasonal anestrous in sheep (Nakao et al., 2008). There is evidence that thyroid hormones affect the normal shape of GnRH neurons in the hypothalamus. The glial processes of these neurons shortens and the body thins which may affect their capacity to secret GnRH (Bernal, 2002; Malpaux, 2006; Nakao et al., 2008). In any event, whether thyroid hormones are involved in the seasonal transition from the ovulatory to the anovulatory season requires further investigation in wood bison.

Using transrectal ultrasonography, follicular dynamics have been found to occur in a wave-like pattern throughout the anovulatory season in wood bison (McCorkell et al., 2013a). In this study, a new wave emergence occurred when the follicle pre-destined to become dominant was 4 mm in diameter. The wave emergence was preceded by a rise in FSH levels, reaching the peak of FSH (approximately 0.35 ng/mL) a day after wave emergence. Selection occurred three days after wave emergence and the dominant follicle reached its maximum diameter of 10 mm three to four days after selection. Because no LH surge occurred, ovulation did not occur and progesterone remained below 1 ng/mL throughout the study. The dominant follicle regressed and new wave emergence occurred. The interval between the emergence of successive dominant follicles was seven days. The pattern of continuous anovulatory follicular waves has also been reported to occur during the entire anovulatory season in other seasonal breeders such as wapiti (McCorkell et al., 2004) and sheep (Rosa and Bryant, 2003).

Our laboratory has also characterized the physiological changes that occur in the bison ovarian function during the transition from the anovulatory to ovulatory season (McCorkell et al., unpublished). The first ovulation occurred in the middle of August, followed by a growth of a small CL (maximum diameter of 16 mm) producing low levels of progesterone (4 to 5 ng/mL in the peak). The CL started to regress six days after the first ovulation and the new dominant follicle ovulated 2.5 days later. Therefore, the interval between the first and second ovulation was 8.5 days. After the second ovulation, progesterone levels were higher (9 to 10 ng/mL) and the interval from the second to the third ovulation was 20 days on average. The mechanism which triggers the first ovulation in bison has not yet been described. However, as in other short-day breeders (e.g., sheep; Robinson et al., 1985), photoperiod and melatonin secretion may be involved in the resumption of the LH surge and ovulation in wood bison.

1.9 Reproductive physiology in cattle

Follicular dynamics in most domestic mammals occur in a wave-like fashion (Driancourt, 2001). In cattle, the presence of two or three consecutive follicular waves occur during the estrous cycle (Ginther et al., 1989). At the time of a wave emergence, there is a recruitment of small antral follicles (approximately of 4 mm of diameter) as a result of the increasing levels of FSH (Adams et al., 2008). A surge of FSH precedes the emergence of a wave (Adams et al., 1992b, Ginther et al., 1996). Selection of the dominant follicle occurs in taurus breeds of cattle when the largest follicle reaches an average of 8 mm in diameter (Ginther et al., 1997; Ginther et al., 2001a). The time of the selection of the dominant follicles and subordinates is called deviation (Ginther et al., 1997). The decline of circulating FSH levels triggers the selection mechanism in cattle (Adams et al., 1993a). During and after selection, the dominant follicle

exerts a suppressive effect on the subordinate follicles and prevents the emergence of a new follicular wave (Adams, 1999; Adams et al., 1993a). To this purpose, the dominant follicle secretes estradiol-17β and inhibin which suppress FSH to a level below than necessary to be used for the subordinate follicles to grow and for the small antral to develop a new wave emergence (Fortune, 1994; Ginther et al., 2000). Although levels of FSH are low, the dominant follicle continues to grow (Adams et al., 1993a). The dominant follicle acquires more LH receptors than the subordinates and, thus, its further development is dependent of LH secretion (Adams et al., 2008; Beg et al., 2001). At the end of the luteal phase, the dominant follicle becomes the preovulatory follicle and, depending on whether the corpus luteum regresses or not, ovulation may occur (Adams and Singh, 2015). If ovulation occurs, the secretion of estradiol ends, the FSH levels increase (which ultimately triggers the emergence of the next wave) and therefore the ovarian cycle is repeated (Edqvist, 1993; Fink, 2000). However, if ovulation does not occur, the preovulatory follicle undergoes atresia and the levels of estradiol decrease (Adams, 1999; Adams and Singh, 2015). Consequently, the FSH rises and a new follicular wave can occur, but not a new cycle. Therefore, induction of a new wave emergence can be achieved by removing the suppressive effect of the dominant follicle on the small follicles (e.g., follicular ablation). This will elicit the rapid increase of FSH levels resulting in the emergence of a new follicular wave (Adams et al., 1992; Bergfelt et al., 1997).

During the luteal phase, follicular waves are under the influence of progesterone until the CL regresses and the next ovulation occurs (Adams and Singh, 2015). Progesterone exerts a negative feedback on the surge of LH and ultimately determines that dominant follicles of successive waves do not ovulate and undergo regression (Adams et al., 1992a). Therefore, the length of the estrous cycle will be determined by the lifespan period of the CL. In a two-wave pattern, the

regression of the CL occurs around Day 16, while in a three-wave pattern the CL begins to regress on Day 19 (Adams, 1999; Adams et al., 2008; Jaiswal et al., 2009). Consequently, the estrous cycle last around 19 - 20 Days in a two-follicular wave pattern cycle whereas it last around 22-23 days in a three-wave pattern (Adams, 1999; Sirois and Fortune, 1988). Therefore, the estrous cycle of 21 days in cattle is the average of the two patterns in cattle.

Recently, it has been suggested that progesterone may also be involved in production of high quality oocytes and embryos (Lonergan, 2011). In *in vitro* studies, progesterone was found to be required for oocyte maturation in cattle (Aparicio et al., 2011). Additionally, progesterone can control the development of the dominant follicle by controlling the LH pulses and preventing early maturation of the oocyte (Lonergan, 2011). Although earlier studies have not found any effect of progesterone levels on embryo quality (Adams et al., 1994a), the effect of progesterone on oocyte and embryo quality must continue to be investigated because the majority of the follicular development occurring during the luteal phase.

1.10 Reproductive technologies to produce healthy embryos from live animals

Reproductive technologies have been developed in order to preserve the genetic material of various wild species (e.g., bison) and domestic animals (Solti et al., 2000). Genetic materials can be preserved in genetic resource banks such as germplasm biobanks (Wildt et al., 1992). Such banks will ultimately allow to storage of semen, oocytes, and embryos for further use in reclamation of an endangered or threatened species (Holt et al, 1996, Wildt, 1992). The advantage of these banks is that the preserved genetics can be maintained indefinitely. In the case of threatened wood bison, technologies such as synchronization of the follicular wave,

superovulation, embryo collection and handling need to be developed to produce disease-free embryos in order to establish a suitable genetic resource bank.

1.10.1 Induction of the follicular wave development

Control of ollicular wave emergence allows researchers to start superstimulatory treatment when small antral follicles are capable of responding to FSH to induce development of multiple follicles (Bo et al., 2002). In cattle, several techniques have been developed to induce emergence of a new follicular wave at a predicted time (Bo et al., 1995a). Follicular ablation or treatment with estradiol-17β + progesterone are the most widely used techniques for synchronizing follicular wave emergence (Mapletoft and Bo, 2015). The aim of these techniques is to remove the suppressive effect of the dominant follicle on FSH release and consequently small antral follicles to allow the emergence of a new follicular wave (Bo et al., 1995a; Bo et al., 2002).

Follicular ablation consists of the physical removal of the dominant follicle which also allows the emergence of a new follicular wave (Bergfelt et al., 1997). Early studies had already shown that the cauterization of the dominant follicle shortened and hastened the emergence of the subsequent follicular wave in cattle (Ko et al., 1991; Adams et al., 1993b). Later, ultrasound-guided aspiration of all follicles ≥ 5 mm of diameter synchronized the emergence of the next wave even though it was used in a random stages of the estrous cycle (Bergfelt et al., 1994). In this study, a surge of FSH occurred a day after follicular ablation and the new follicular wave emerged at Day 1.5. Follicular ablation was also used to synchronize follicular wave emergence in cows for superovulation (Bergfelt et al., 1997; Baracaldo et al., 2000). The technique had been developed for transvaginal oocyte collection in cattle (Pieterse et al., 1988; 1991). In bison,

follicular ablation was also used to synchronize follicular wave emergence. Based on the same methodology developed in cattle, follicular ablation induced the emergence of a new follicular wave in wood bison 1 day after the aspiration of all follicles ≥ 5 mm of diameter during the ovulatory season (McCorkell et al., 2010) as well as the anovulatory season (Palomino et al., 2014a). Additionally, the emergence of the follicular wave was more synchronous than following any other treatments (Palomino et al., 2014a) and has been used successfully recently in a superovulatory protocol in wood bison (Toosi et al., 2013). The advantage of follicular ablation in wood bison is that it can be done rapidly if performed by trained personnel and if the animals do not need to be sedated. The disadvantage of this technique is that it requires specialized equipment (i.e., a transvaginal probe and ultrasound machine) and restraint facilities (i.e., hydraulic chute) that may not be available in the field.

Estradiol-17β along with progesterone have been used to induce a new follicular wave in cattle (Bo et al., 1995a). A single injection of 5 mg of estradiol-17β in cattle was effective in inducing follicular suppression and synchronous emergence of a new follicular wave 4 to 5 d later (Bo et al., 1994a). The action of the estradiol in synchronizing follicular wave emergence is not well understood, but it was reported that estradiol suppresses levels of FSH, thereby preventing the growth of FSH-dependent follicles (Adams, 1999). Likewise, when progesterone was added to the treatment with estradiol-17β, a synchronous emergence of the next follicular wave occurred four days later (Bo et al., 1994a; Bo et al., 1995b). The suppressive effect of the progesterone on LH pulses appears to suppress the growth of LH-dependent follicles, enhancing the action of estradiol (Adams et al., 1992a; Bo et al., 1995b). In wood bison, 5 mg of estradiol-17β during the anovulatory season has induced a new follicular wave 3 days after treatment, but unintended ovulation occurred after estradiol administration in 43% of treated females

(McCorkell et al., 2010). In a follow up study, the administration of a single dose of estradiol-17β (2.5 mg) + progesterone (50 mg) resulted in the emergence of the new wave at 4.1 days after onset of treatment, and no ovulation after synchronization was reported (Adams et al., 2010). Recently, the combination of estradiol-17β (2.5 mg) + progesterone (50 mg) induced a new follicular wave 3.2 days after administration of steroid hormones (Palomino et al., 2014a). The advantage of this technique is that a single injection of steroid hormones can synchronize new wave emergence. Therefore, several animals can be synchronized at once.

1.10.2 Superovulation and embryo collection

A necessary step in establishing the germplasm bio-bank is the ability to produce high quality embryos from endangered species (Wildt, 1992). Superovulation and embryo collection are technologies that have been used to produce embryos in wild animals (Comizzoli et al., 2000) and domestic livestock (Hansen 2014). The goal of a superovulatory protocol is to obtain the maximum number of transferable embryos with high likelihood of obtaining a pregnancy (Nasser et al., 1993; Mapletoft et al., 2002). The technique has been developed in cattle as part of an embryo transfer program with the ultimate goal of improving genetics (Seidel, 1981). Superovulatory treatments rescue the subordinate follicles that otherwise would regress due to the suppressive effect of the dominant follicle (Adams et al., 1993b), allowing them to grow until they reach an ovulatory size (Gordon, 2004). Therefore, the superstimulatory treatment must be initiated at the time of follicular wave emergence to avoid the suppressive effect of the dominant follicle on subordinate follicles (Adams, 1994; Nasser et al., 1993). Intrinsic (i.e., age, nutritional status, reproductive history, ovarian status, etc.) and extrinsic (type of gonadotropin used, dose, treatment scheme, season, environment, etc.) factors have been shown to be

involved in the success of the superovulatory protocols in cattle (Kafi and McGowan, 1997; Mapletoft et al. 2002). These factors are responsible for the extreme variability of the superstimulatory response and must be taken into account to establish an efficient protocol for superstimulation in any species.

Two hormones have been used to stimulate multiple follicle development (superstimulation): eCG and FSH (Goulding et al., 1991; Mapletoft et al., 2002). Equine chorionic gonadotropin (eCG) is a glycoprotein of around 150 aminoacids which is secreted by the endometrial cups of the equine placenta (Murphy and Martinuk, 1991). The use of eCG to induce ovarian superstimulation has been discontinued due to its low superovulatory results in cattle from a high number of anovulatory follicles (Monniaux et al., 1983; Sendag et al., 2008). This response may be related to the long half-life of eCG which may be around 3 to 5 days in cattle (Murphy and Martinuk, 1991). Conversely, follicle-stimulating hormone (FSH), a glycoprotein secreted by the anterior pituitary, has also been used to induce ovarian superstimulation in cattle (Gonzalez et al., 1990; Mapletoft et al., 2002). The traditional scheme of superovulation includes administration of FSH twice a day for four or five days (Mapletoft et al., 2002). Twice daily treatments are considered necessary because the half-life of the FSH in the cow has been reported to be approximately 5 hours (Laster, 1972). Over the years, FSH has been used in superovulatory protocols and played a key role in the improvement of embryo transfer programs in cattle (Bo and Mapletoft, 2014).

In bison, the first attempts at superovulation involved the use of bovine protocols, with limited results (Dorn et al., 1990, Robison et al., 1998; Othen et al., 1999). In an early study, 14 bison cows were superovulated using the multiple dose of FSH regime developed for cattle, but only 5 transferable embryos were collected (Dorn et al., 1990). A later study obtained 1 corpus

luteum and no embryos in bison cows superovulated with the multiple dose protocol of FSH (Othen et al., 1998). These modest results have been attributed to the stress associated with handling, mainly due to the twice daily FSH treatment (Dorn, 1995). To overcome this problem, the Dorn et al. suggested the use of eCG as a single dose to induce superovulation in bison. However, superovulation with a single dose of eCG in 16 plains bison cows resulted in a mean of 2 corpora lutea and 0.6 transferable embryos (Robison et al., 1998). In our laboratory eCG induced a low number of follicles ≥ 5 mm in wood bison during the anovulatory season (8.1 follicles; Palomino et al., 2013) and ovulatory season (5.6 follicles; Palomino et al., 2014b). Therefore, eCG may not be ideal to induce superstimulation in wood bison and a different method or a more simplified FSH regime needs to be investigated.

In cattle, single and double subcutaneous injections of FSH have been used to induce ovarian superstimulation and the results were comparable to those obtained in the traditional twice-daily protocol of FSH (Bo et al., 1994b; Lovie et al., 1994; Alvarez et al., 2010). Considering the stress associated with twice daily injections, these less-frequent superstimulatory protocols may be more useful in wild species (e.g., bison) that are difficult to handle on a frequent basis. Using the above approaches, two subcutaneous injections of FSH given behind the shoulder increased the number of follicles ≥ 5 mm in wood bison during the anovulatory season (14.6 follicles; Palomino et al., 2013) and the ovulatory season (12.2 follicles; Palomino et al., 2014b). However, the superovulatory response to a double subcutaneous injection of FSH is dependent on sufficient subcutaneous adipose tissue to slow the absorption of FSH (Bo et al., 2010). Therefore, single or double subcutaneous FSH injections may not work well in wood bison with less fat tissue under the skin.

Later, a single or double intramuscular injection FSH protocol involving the use of hyaluronan as a diluent have been developed for use in cattle (Bo et al., 2010). There was no difference in superovulatory response when a single injection of FSH in 2% hyaluronan or two doses of FSH diluted in 0.5 or 1% hyaluronan were compared to the twice daily intramuscular injection schedule in cattle (Tribulo et al, 2011; 2012). Recent studies in wood bison have demonstrated that these superovulation protocols are feasible in bison and that live wood bison offspring can be obtained (Toosi et al., 2013). In this study, superovulation was accomplished by using a single subcutaneous injection of FSH and response was not different from two injections of FSH given subcutaneously. The challenge now is to improve the superovulatory response and embryo quality using these simplified methods of superstimulation in wood bison.

Non-surgical embryo collection has been described previously in cattle (Rowe et al., 1976; Elsden et al., 1976; Greve et al., 1977; Newcomb et al., 1978). Since then, several modifications of the technique have been made to improve the efficiency of embryo collection. In general, the technique uses a 2 or 3-way catheter that is introduced into the uterus or one uterine horn through the cervix with the help of a stainless steel stylet. Once in place, a balloon located near the tip of the catheter is inflated to fix the catheter in the uterus and prevent reflux of the flushing medium. The uterus is then flushed with a isotonic buffer medium. This wash is returned through the same catheter and retrieved with syringes or collected directly in embryo filters. Embryos are searched at 10X magnification under a stereomicroscope using 90 mm Petri dishes (Rowe et al., 1979; Mapletoft, 1986, Stringfellow and Givens, 2010). In bison, the non-surgical technique has been previously applied (Dorn et al., 1995). Recently, transferable embryos were collected from live wood bison without sedation and five good (IETS code 1) quality embryos were transferred directly into synchronized recipients (Toosi et al., 2013). This

resulted in the birth of the first two wood bison calves that come from embryo transfer. Therefore, the nonsurgical approach for embryo collection and transfer can be safely used in wood bison

1.10.3 Production of disease-free embryos

Although the advent of reproductive technologies has helped prevent dissemination of animal disease, embryos will always be considered a biohazard for the transmission of pathogens in embryo transfer programs (Givens et al., 2007). Therefore, washing procedures have been developed to reduce the risk of transmission of pathogens through embryo transfer technologies (Bielanski, 2007; Givens and Marley, 2008). There are different potential sources of contamination of in vivo-produced embryos (Bielanski, 2007). Some viruses (i.e., bovine herpesvirus-1 [BHV-1], bovine viral diarrhea virus [BVDV]) can contaminate the oocyte in the follicular fluid, and consequently, the embryo (Bielanski and Dubuc, 1994; Brownlie et al., 1997). Embryos can also be contaminated in the oviduct or uterus of an infected female (Booth et al., 1995; Xavier et al., 2009). Finally, embryos may be infected with pathogens from the environment during collection, manipulation, cryopreservation, or transfer (Bielanski and Stewart, 1996; Bielanski et al., 2003; Givens and Marley, 2008). Embryos in early stages are surrounded by the zona pellucida (Green, 1997). The zona pellucida (ZP) is a glycoprotein layer composed of three proteins: ZP1, ZP2, and ZP3 (Wassarman, 1988). This shell plays an important role during the fertilization and protects the embryo from internal and external environmental factors (Green, 1997). An intact ZP is an effective barrier against most pathogens and prevents embryo infection (Van Soom, 2010; Stringfellow and Givens, 2010). Therefore, to

perform effective washing procedures, the ZP must be intact (not be damaged or broken) and free of adherent material (Stringfellow and Givens, 2010).

Washing procedures for rendering embryos free of pathogens have been developed in cattle and are described in the Manual of the International Embryo Transfer Society (Stringfellow and Givens, 2010). The procedures consist of the transfer of embryos through ten successive wells which contain wash medium (PBS + 0.4% BSA) plus antibiotics (i.e., 100 IU/mL penicillin and 100 μg/mL streptomycin). A 100 fold dilution must occur and a new sterile pipette must be used for each wash. Specific guidelines must be followed during the procedures to ensure the removal of the pathogens (Givens and Marley). For instance, no more than ten embryos can be washed at once, dilution factor must be 1:100. Trypsin treatments may be added in the middle of the washing procedures when suspect of viral infection because viruses may stick to the zona pellucida. Additionally, embryos with damaged ZP must be discarded (Stringfellow and Givens, 2010). Disinfected embryos are considered specified pathogen free and can be transferred or cryopreserved in a liquid nitrogen.

Although the transmission of *Brucella* bacteria through the reproductive system (i.e., the uterus) has been the subject of debate for many years (Del Campo et al., 1987), there is risk of transmission of the pathogen due to its presence in the uterine tissue in pregnant cows (Forbes and Tessaro, 1996; Xavier et al., 2009). In these studies, the bacteria were detected in endometrial tissue and lymph nodes associated with the uterus (e.g., internal iliac). Interestingly, in naturally infected non-pregnant cows, the pathogen was also found in the uterus and associated local lymph nodes (Gallien et al., 1998). The fact that *Brucella* bacteria were present in all tissues described above may suggest that the pathogen has potentially capacity to infect embryos located in the uterus of infected animals and may be transmitted to healthy recipients by

embryo transfer techniques (Stringfellow and Givens, 2000). Whether the *Brucella* bacterium is transmitted attached to the embryo or through the uterine fluids, needs further investigation.

CHAPTER 2. GENERAL HYPOTHESIS

Superovulation and embryo collection will be enhanced by tailoring treatment protocols to the endogenous rhythm of ovarian function in wood bison.

CHAPTER 3. GENERAL OBJECTIVES

1. Develop effective methods of superovulation and collection of multiple embryos from bison donors to mitigate cattle disease transmission and preserve genetic diversity.

2. Develop protocols that are sufficiently precise to allow artificial insemination and embryo collection at self-appointment time

CHAPTER 4

INDUCING OVULATION IN WOOD BISON (BISON BISON ATHABASCAE) DURING THE ANOVULATORY SEASON

Relationship of this study to the dissertation:

Inducing ovulation during the anovulatory (i.e., non-breeding) season is a necessary prelude for protocol development for superovulation and embryo collection during this period. However, there are few studies done in seasonal breeders regarding gonadotropin treatments to induce ovulation during the anovulatory season. Therefore, there was a need to investigate whether ovulation can be induced during the anovulatory season in wood bison. Results from this study were very important to learn about the feasibility of induction of ovulation in wood bison out of the breeding season and to determine the size of the dominant follicle that responded optimally to gonadotropin treatment. These factors are crucial for developing protocols of superovulation in this species.

4.1. Abstract

As part of the development of a germplasm biobank to preserve the genetic diversity of threatened wood bison (*Bison bison athabascae*), a 2x2 factorial study was designed to determine the effects of ovulation induction agent and follicle maturity on the ovulatory response in wood bison during the anovulatory season. Bison (n=32) were assigned randomly to four groups (n=8/group) and treated with either pLH or hCG when a growing dominant follicle was either 8-9 mm or ≥10 mm. The ovaries were examined daily by ultrasonography to determine the timing of ovulation, and 7 days post-treatment to assess CL development. The proportion of bison that ovulated was greater in bison treated with hCG than pLH ([15/16] 94% vs. [8/16] 50%; $P<0.05$), and when the dominant follicle was ≥10 mm vs. 8-9 mm at the time of treatment (88% vs 56%; $P<0.05$). The interval from treatment to ovulation was 37.0 ±1.3 hours and was not affected by induction agent or follicle size. However, synchrony of ovulation tended to be greater ($P = 0.10$) in the ≥10 mm group vs. the 8-9 mm group, and the ensuing corpus luteum was larger (15.3 ± 0.43 mm vs. 13.4 ± 0.36; $P<0.05$). In conclusion, both ovulation inducing agent and follicle size influenced the ovulatory response in bison during the anovulatory season. Treatment with hCG was more effective than pLH for inducing ovulation in wood bison, and the effect was greater when treatment was given when the growing dominant follicle was ≥10 mm.

4.2. Introduction

As the largest land mammal in North America, the wood bison (*Bison bison athabascae*) has played a prominent role in the culture and history of First Nation communities in Canada (Garret, 2007), and in maintaining the balance of the boreal forest ecosystem (Sanderson et al., 2007). However, the wood bison has been designated a threatened species (Species at Risk Act, 2002), with a current population of <6% of historic numbers (COSEWIC, 2013). The population in

Wood Buffalo National Park in Canada represents the largest reserve of wood bison in the world, but is endemically infected with bovine tuberculosis and brucellosis (McCormack, 1992). These diseases retard population growth, are a reservoir for infection of healthy bison and other animals in and around the park, and hamper efforts to conserve the species (McCormack, 1992; Mitchell and Gates, 2002). To retain the genetic diversity of wood bison and to mitigate the effects of endemic diseases, we propose the use of reproductive technologies to generate healthy replacements through the use of disease-free germplasm as a viable method of wood bison conservation.

Induction of synchronous ovulation among individuals has facilitated the use of fixed-time artificial insemination in cattle (Martinez et al., 1999; Colazo et al., 2009; Pursley et al., 1995) and embryo transfer technologies in cattle (Baruselli et al., 2011; Bergfelt et al., 1997; Bo et al., 2006). Few studies, however, have been reported in bison (Dorn, 1995) and development of effective protocols has been hampered by the lack of information regarding the basic reproductive physiology of this species. Recently, the normal reproductive pattern in female wood bison was characterized using serial ovarian ultrasonography during the anovulatory season (McCorkell et al., 2013). Follicular development was characterized by periodic emergence of a group of follicles growing at a similar rate, followed within a few days by selection of one dominant follicle which continued to grow while the others (subordinates) regressed (i.e., wave-like pattern; Ginther et al., 1989). The dominant follicle did not ovulate, but rather regressed and the wave pattern repeated itself at 7-day intervals. In cattle, the diameter of the ovulatory follicle ranged between 12 and 15 mm (Perry et al., 2007); however, the dominant follicle attained the capacity to ovulate at 10 mm, corresponding to the day after divergence in the growth profile between the dominant versus subordinate follicles (Sartori et al., 2001). In

cattle, the effectiveness of ovulation induction treatment was found to be related to the size of the dominant follicle at the time of treatment (Martinez et al., 1999). In wood bison during the anovulatory season, divergence in the growth profiles of the dominant and largest subordinate follicle occurred approximately 3 days after wave emergence, when the dominant follicle was 6–7 mm (McCorkell et al., 2013). Although no data have been reported about when ovulatory capacity is attained in bison, we anticipated that a dominant follicle of 8 mm in diameter (i.e., the day after divergence in dominant-subordinate growth profiles) would be sufficiently mature to ovulate in bison based on previous work on ovarian superstimulation in this species (Palomino et al., 2013; 2014b).

Gonadotropin releasing hormone (GnRH), or its agonists, is the most extensively used drug to induce ovulation in cattle (Thatcher et al., 1993; Pursley et al., 1995), but others include porcine LH (pLH) and human chorionic gonadotropin (hCG; Lamb et al., 2010). In one study (Ree et al., 2009), the administration of 25 mg of pLH in non lactating cows induced a greater proportion of cows to ovulate than 100 μg of GnRH. Others have used hCG to induce ovulation of the dominant follicle of the first wave in cattle to produce an accessory corpus luteum (Diaz et al., 1998; Santos et al., 2001), and the administration of 3000 IU of hCG resulted in a similar percentage of ovulation to that of 100 μg GnRH (Marquezini et al., 2011). To date, the ovulation inducing effects of GnRH, pLH, and hCG has not been critically compared in seasonal breeders, including bison. In the mare, the effectiveness of GnRH during the anovulatory season was dependent on prolonged administration through repeated doses (Ginther and Bergfelt, 1990; Mumford et al., 1994); however, the incidence of ovulation was not high enough to determine its value for fixed-time artificial insemination. Since pLH and hCG act directly on the receptors on

ovarian follicles (Ascoli et al., 2002), they may be a more effective alternative to induce ovulation in seasonal breeders during the anovulatory season.

Reliable induction of ovulation is a prerequisite for the development of protocols for timed artificial insemination, either for singleton pregnancy or for superovulation and embryo collection. The objective of this study was to determine the effects of dominant follicle maturity and ovulation-inducing agent on the ovulatory response in wood bison during the anovulatory season.

4.3. Materials and methods

4.3.1. Animals

Adult female wood bison (n=32) between 6 and 10 years of age, with an average body condition score of 3.5 (BSC scale of 1 [thin] to 5 [fat]; Vervaecke et al., 2005), were used during the anovulatory season (June). The bison were maintained at the Native Hoofstock Centre, University of Saskatchewan, Saskatoon, Canada, and had free access to alfalfa/rye grass hay and fresh water, and were supplemented with approximately 1.5 kg/head/day of oats. The experimental protocol was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

4.3.2. Experimental design

Bison were assigned randomly to four groups (n=8/group) using a 2 x 2 factorial design to compare the ovarian response to treatment with pLH versus hCG when the dominant follicle was 8-9 mm versus ≥10 mm. The ovaries were examined once per day by transrectal

ultrasonography using a 7.5 MHz linear-array transducer (Aloka SSD 900, Tokyo, Japan), as previously described in bison (McCorkell et al., 2013; Palomino et al., 2014a). Briefly, the bison were restrained in a squeeze-chute in the standing position without sedation, and the ultrasound transducer was placed into the rectum using a gloved hand. All follicles ≥4 mm in both ovaries were sketched on a diagram of the ovary to record size, number, and location. The three largest follicles were individually identified and monitored through growth and regression. Follicular wave emergence (designated Day 0) was defined as the day when the follicle destined to become dominant in the wave was first detected at a diameter of 4-5 mm (Ginther et al. 1989; McCorkell et al., 2013). A single intramuscular dose (based on manufacturer's recommendation for use in cattle) of either pLH (25 mg Lutropin-V, Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada) or hCG (2500 IU Chorulon, Merck Animal Health, Kirkland, Quebec, Canada) was given when the growing dominant follicle was 8-9 mm or when first detected at a diameter of ≥10 mm, in the respective groups. The ovaries were examined by ultrasonography every 12 hours post-treatment until ovulation, or for three days, whichever occurred first. Ovulation was defined as the disappearance of the dominant follicle from one examination to the next. The ovaries were examined again 7 days after treatment to determine the presence and size of the corpus luteum.

4.3.3 Statistical analyses

Preovulatory follicle diameter, interval to ovulation after treatment, and CL diameter were compared among groups by two-way analysis of variance and Tukey's post hoc tests. Normality was assessed with Shapiro-Wilk test. The proportion of bison that ovulated (number of bison that ovulated/number of bison in group) was analyzed by logistic regression. The degree of ovulation

synchrony was compared by two-way analysis of variance of residuals (i.e., the absolute difference between the group mean and individual values). A probability of ≤0.05 was considered statistically significant. Data analysis was done using SAS version 9.2 (Cary, NC, USA). Data are presented as mean ± SEM, unless otherwise indicated.

4.4. Results

The interval (mean ± SEM) from the day of wave emergence to the day of treatment was 3.8 ± 0.14 and 4.9 ± 0.17 days for bison treated when the dominant follicle was 8-9 mm and ≥10 mm, respectively (P<0.05). Data are summarized in Table 1. As expected from the experimental design, the diameter of the dominant follicle at the time of treatment with either pLH or hCG was greater in the ≥10 mm group than in the 8-9 mm group (P<0.05). There was, however, no difference among groups in the diameter of the dominant follicle 12 h before ovulation as a result of more rapid growth in the 8-9 mm group vs ≥10 mm group between the time of treatment and ovulation (1.8 mm vs 0.6 mm; P<0.05).

Table 4.1. Response (mean ± SEM) to ovulation induction treatment in wood bison during the anovulatory season.

	Dominant follicle (DF) 8 - 9 mm	Dominant follicle (DF) ≥ 10 mm	Combined
Follicle diameter at treatment (mm) [1]			
pLH	8.8 ± 0.2 (n = 8)	11.6 ± 0.3 (n = 8)	10.2 ± 0.4 (n = 16)
hCG	8.9 ± 0.2 (n = 8)	11.4 ± 0.1 (n = 8)	10.1 ± 0.3 (n = 16)
Combined	8.9 ± 0.1 (n = 16)	11.5 ± 0.2 (n = 16)	10.2 ± 0.3 (n = 32)
Follicle diameter before ovulation (mm) [2]			
pLH	11.0 ± 1.0	11.6 ± 0.5 (n = 6)	11.4 ± 0.4 (n = 8)

	(n = 2)		
hCG	10.7 ± 0.4 (n = 7)	10.8 ± 0.3 (n = 8)	10.8 ± 0.2 (n = 15)
Combined	10.8 ± 0.3 (n = 9)	11.1 ± 0.2 (n = 14)	11.0 ± 0.2 (n = 23)
Ovulation rate (# of bison that ovulated/ # of bison in group) [3]			
pLH	2/8 (25%)	6/8 (75%)	8/16 (50%)
hCG	7/8 (88%)	8/8 (100%)	15/16 (94%)
Combined	9/16 (56%)	14/16 (88%)	23/32 (72%)
Interval from treatment to ovulation (hours) [4]			
pLH	42.0 ± 6.0 (n = 2)	38.0 ± 2.0 (n = 6)	39.0 ± 2.0 (n = 8)
hCG	41.1 ± 2.4 (n = 7)	36.0 ± 2.8 (n = 8)	38.4 ± 1.7 (n = 15)
Combined	41.4 ± 2.1 (n = 9)	36.9 ± 1.5 (n = 14)	37.0 ± 1.3 (n = 23)
Variation in interval from treatment to ovulation (residuals, hours) [5]			
pLH	6.0 ± 0.0 (n = 2)	3.3 ± 1.3 (n = 6)	4.0 ± 1.1 (n = 8)
hCG	5.9 ± 0.4 (n = 7)	3.0 ± 2.0 (n = 8)	4.3 ± 1.1 (n = 15)
Combined	5.9 ± 0.3 (n = 9)	3.1 ± 1.2 (n = 14)	4.2 ± 0.8 (n = 23)
Corpus luteum diameter (mm) 7 days after treatment [6]			
pLH	12.5 ± 0.5 (n = 2)	14.8 ± 0.6 (n = 6)	14.3 ± 0.6 (n = 8)
hCG	13.7 ± 0.6 (n = 7)	15.6 ± 0.7 (n = 8)	14.7 ± 0.5 (n = 14)
Combined	13.4 ± 0.5 (n = 9)	15.3 ± 0.5 (n = 14)	14.6 ± 0.3 (n = 23)

[1] DF 8-9 mm vs ≥10 mm ($P < 0.05$), pLH vs hCG ($P = 0.88$), Interaction ($P = 0.28$)
[2] DF 8-9 mm vs ≥10 mm ($P = 0.63$), pLH vs hCG ($P = 0.09$), Interaction ($P = 0.53$)
[3] DF 8-9 mm vs ≥10 mm ($P < 0.05$), pLH vs hCG ($P < 0.05$), Interaction ($P = 0.17$)
[4] DF 8-9 mm vs ≥10 mm ($P = 0.11$), pLH vs hCG ($P = 0.56$), Interaction ($P = 0.85$)
[5] DF 8-9 mm vs ≥10 mm ($P = 0.10$), pLH vs hCG ($P = 0.88$), Interaction ($P = 0.96$)
[6] DF 8-9 mm vs ≥10 mm ($P < 0.05$), pLH vs hCG ($P = 0.51$), Interaction ($P = 0.79$)

The proportion of bison that ovulated varied from 25% to 100% among groups, and was greater in bison in the larger follicle diameter group ($P < 0.05$) and in those treated with hCG rather than pLH ($P < 0.05$); there was no interaction of main effects (Table 1). The interval from treatment to ovulation was not affected by induction agent (pLH vs hCG) but tended to be longer in bison treated when the dominant follicle was 8-9 mm than when ≥10 mm ($P = 0.11$; Table 1). A corpus luteum was detected 7 days after treatment in only those bison in which ovulation was detected previously by ultrasonography (taken as the sudden disappearance of the largest follicle from one examination to the next). That is, no ovulations occurred >3 days after treatment. The interval from treatment to ovulation ranged from 24 to 48 hours; all but one (22/23; 96%) ovulated between 36 and 48 hours after treatment. Analysis of residuals, as an indication of the degree of synchrony in ovulation, revealed no significant differences among groups; however, synchrony tended to be greater in bison treated when the dominant follicle was ≥10 mm than when 8-9 mm ($P = 0.10$; Table 1).

The diameter of the corpus luteum that developed subsequent to induction of ovulation was not affected by induction agent (pLH vs hCG), but was greater in bison treated when the dominant follicle was ≥10 mm in diameter than when 8-9 mm ($P < 0.05$; Table 1).

4.5. Discussion

In the present study, ovulation and CL development were induced during the anovulatory season in 72% of wood bison, and ovulations within each group occurred within a 12-hour window in all but one bison (i.e., 96%). Treatment with hCG was nearly twice as effective as pLH for inducing ovulation (94% vs 50%). As well, the maturity of the dominant follicle at the time of the gonadotropin treatment, reflected by its diameter, was positively associated with

ovulation rate and the size of the ensuing corpus luteum. Treatment with hCG when the dominant follicle was ≥10 mm resulted in an ovulation rate of 100% in 36 h.

Like all gonadotropins, LH and hCG are glycoproteins that have a common α subunit, but a differing β subunit that confers their specific biological activity (Choi and Smitz, 2014; Pierce and Parsons, 1981). Both LH and hCG induce ovulation and luteinization by binding to the same receptor (LH/hCG receptor) on ovarian follicular cells (Ascoli et al., 2002), However, the β subunit of hCG contains 24 amino acids more than that of LH (Talmadge et al., 1984) which imparts a longer half-life and an increased potency (around 5-fold greater) than LH (Choi and Smitz, 2014). When given in equipotent doses (same effective dose), hCG induced greater cAMP production (principal second messenger) in cultured granulosa cells than LH (Casarini et al., 2012). The circulating half-life of the LH is approximately 30-60 minutes (Robertson et al., 1991), whereas that of hCG is approximately 37 hours (Faiman et al., 1968, Schmitt et al., 1996). The longer half-life may allow hCG longer occupancy on the LH/hCG receptors than LH (Matzuk et al., 1990, Filicori et al., 2005).

No previous reports were found about inducing ovulation with hCG in wood bison, but in cattle, hCG has been used to induce ovulation in timed artificial insemination programs (Stevenson et al., 2007; De Rensis et al., 2010). A greater proportion of cows ovulated after treatment with hCG than with GnRH (Dahlen et al., 2011), but no difference was found between cows given hCG vs. pLH (Gordon, 2011). The difference may be attributed to an indirect versus direct effect of inducing a preovulatory LH surge using GnRH versus exogenous LH or hCG. Both LH and hCG act directly on the ovarian follicles (Yavas et al., 1999).

A greater proportion of bison ovulated when, at the time of treatment, the dominant follicle was ≥10 mm (88%) than when 8-9 mm (56%). We anticipated that a dominant follicle of 8 mm

would be mature enough to ovulate in wood bison; however, it appears that full ovulatory capacity in this species may not be attained until the dominant follicle reaches a diameter of 10 mm. In cattle, ovulatory capacity was apparent when the dominant follicle was 10 mm, but a greater number of ovulations occurred in response to treatment when the dominant follicles were 12-13 mm (Sartori et al., 2001). The change in ovulatory capacity during growth of the dominant follicle is related to acquisition of LH receptors; the number of LH-receptors per follicle was three times higher in 13 mm follicles compared to 8-10 mm follicles (Bodensteiner et al., 1996). Results of the present study are consistent with the hypothesis that larger follicles (i.e, ≥10 mm of diameter) possess a greater number of LH/hCG receptors, and are thereby more capable of ovulation in response to the gonadotropin signal.

All ovulations occurred between 24 and 48 hours after treatment (mean 37 hours) in the present study. However, ovulation occurred 4.5 h sooner in bison treated when the dominant follicle was ≥10 mm than when 8-9 mm (36.9 h vs 41.4 h respectively; P = 0.11). The rapid growth rate in the 8-9 mm group and nearly flat growth rate in the ≥10 mm group is consistent with the concept that maturation is not complete in the former and full ovulatory capacity has not been attained; i.e., immature follicles need more time to grow and acquire sufficient LH receptors (Sartori et al., 2001; Bodensteiner et al., 1996). Interestingly, the slowing of the growth rate of the maturing dominant follicle is particularly evident in prepubertal heifers nearing their first ovulation (Adams et al., 1994; Evans et al., 1994) and in bison in the transition to the ovulatory season (McCorkell et al., 2013).

Luteinization occurs in the theca and granulosa layers of the follicular wall after ovulation (Richards et al., 1998), and LH/hCG promotes the development of the CL (Farin et al., 1988, Ree et al., 2009). In cattle, ovulation of a small dominant follicle has been shown to be associated

with subsequent development of a small CL that secretes less progesterone (Vasconcelos et al., 2001). Conversely, higher circulating concentrations of progesterone were found in cattle after ovulation of a large follicle (Perry et al., 2007). Results of the present study are consistent with the concept of a positive relationship between the diameter of the ovulatory follicle and the form and function of the ensuing CL; wood bison induced to ovulate when the dominant follicle was ≥10 mm developed a larger CL than when the dominant follicle was 8-9 mm. This finding may have important implications for fertility subsequent to induced ovulation in bison since the pregnancy rate was lower in cows that ovulated a small (<11 mm) dominant follicle (Perry et al., 2005, Perry et al., 2007).

In summary, both the ovulation agent and follicle size influenced the ovulatory response in bison during the anovulatory season. Treatment with hCG induced nearly twice as many ovluations as pLH, and the effect was greater when treatment was given when the growing dominant follicle was ≥10 mm. The maturity of the dominant follicle at the time of the gonadotropin treatment, reflected by its diameter, was positively associated with the proportion of bison that ovulated and the size of the ensuing corpus luteum. Future work will focus on the effects of induction agent and follicle maturity on the developmental competence of oocytes fertilized in vivo or in vitro during the anovulatory season.

4.6. Acknowledgements

The authors thank Nick Hawkins for assistance with data collection, Bioniche Animal Health Canada Inc. for providing Lutropin-V, and Merck Animal Health for providing Chorulon. We thank Elk Island National Park for donating the bison that now comprise the research herd at the Native Hoofstock Centre, University of Saskatchewan. We also thank Dr. Murray Woodbury,

Agriculture and Agri-Food Innovation Fund Specialized Livestock Research Chair at the Western College of Veterinary Medicine, for fostering the bison research program and for providing professional herd management and oversight.

CHAPTER 5.

SUPEROVULATION IN WOOD BISON (*BISON ATHABASCAE)* DURING THE OVULATORY AND ANOVULATORY SEASONS: EFFECTS OF PROGESTERONE, TREATMENT PROTOCOL AND GONADOTROPIN PREPARATIONS FOR THE INDUCTION OF OVULATION

Relationship of this study to the dissertation:

First attempts to develop superovulation protocols in bison resulted in low ovarian response. The stress of handling due to multiple FSH treatments was considered to be the main factor associated with the low superstimulatory response. Therefore, simplified methods of superstimulation in wood bison cows were utilized to minimize the stress of handling. Although simplified FSH protocols appeared to decrease stress, the ovarian response was still low. Thus, we attempted to improve the ovarian response and embryo quality in wood bison by modifying the simplified FSH regime. Additionally, we investigated the use of hCG to induce ovulation in superstimulated cows. Results of this study were important in optimizing FSH treatment and ovulation-induction treatment regime in bison. Results were used to further improve superovulatory protocols in subsequent studies in this thesis (Chapter 6 and 7).

5.1 Abstract

Experiments were done to determine the ovarian response and embryo production following superstimulation of wood bison during the anovulatory and ovulatory seasons. In the anovulatory season (Experiment 1), the efficacy of pLH vs. hCG for the induction of ovulation was compared in wood bison superstimulated with a single dose of pFSH in 0.5% hyaluronan and the effect of exogenous progesterone (PRID) on superovulatory response and embryo quality was examined. In the ovulatory season (Experiment 2), the efficacy of pLH vs. hCG for the induction of ovulation was compared in wood bison superstimulated with pFSH in 0.5% hyaluronan as a single intramuscular dose vs. a two-dose regimen 48 hours apart (split dose). In Experiment 1, the number of CL was greater ($P<0.05$) in bison treated with hCG than pLH, (6.6 ± 1.8 vs. 2.8 ± 0.8, respectively). There was no effect of progesterone treatment on embryo quality. In Experiment 2, the number of CL was greater ($P < 0.05$) in bison treated with hCG than with pLH (6.3 ± 0.8 vs. 3.8 ± 1.2, respectively) and in bison superstimulated with split dose vs. single dose of FSH (7.1 ± 0.9 vs. 3.0 ± 0.8, respectively). The number of ova/embryos and freezable embryos did not differ among groups in either experiment. In conclusion, hCG induced a greater ovulatory response than pLH in both the oanovulatory and ovulatory seasons. The split dose of FSH induced a greater superovulatory response in wood bison during the ovulatory season. Exogenous progesterone did not improve fertilization rates or embryo quality during the anovulatory season.

5.2. Introduction

The largest reserve of wood bison (*Bison bison athabascae*) in the world resides in Canada's Wood Buffalo National Park, and herds within the park represent the greatest source of genetic

diversity of this threatened species (Wilson et al., 2005; COSEWIC 2013). At only 6% of their estimated historic population of 168,000 (Joly and Messier, 2001), further loss of genetic diversity threatens the ability of extant wood bison to adapt to environmental changes or survive adverse stochastic events, and may lead to extinction (Frankham 2005; McFarlane et al., 2006). Recovery of this genetic resource, however, is complicated by the existence of endemic tuberculosis and brucellosis within herds in Wood Buffalo National Park. These zoonotic diseases have been implicated in the lack of population growth of wood bison and, perhaps more importantly, resulted in regulations that prevent their expansion into former ranges outside the Park (Mitchell and Gates, 2002; Shury et al., 2015).

Superovulation and embryo transfer are technologies that may be used for reclamation of threatened and endangered species (Wildt, 1992; Holt et al., 1999; Loskutoff et al., 1995; Solti et al, 2000). In bison, the first attempts at superovulation involved the use of bovine protocols, but results were disappointing (Dorn et al., 1990, Robison et al., 1998; Othen et al., 1999). In one study, 14 plains bison were treated with FSH to induce superovulation and only 5 transferable embryos were collected (Dorn et al., 1990). These modest results were attributed to a poor superstimulatory response, likely associated with the stress of handling (Dorn, 1995). In a later study, however, the superovulatory response did not differ in plains bison treated with multiple FSH treatments vs. a single treatment with eCG (Othen et al., 1998). On average, one CL per cow was found in both groups (n = 5 bison/group), but embryo collection was not done. In another study in plains bison (Robison et al., 1998), a single dose of eCG (n = 16) resulted in a mean of 2 CL and 0.6 transferable embryos per animal treated. More recently, we found that the superstimulatory response (i.e. number of follicles >5 mm at the end of treatment) in wood bison treated with eCG was approximately half that of bison treated with FSH during the ovulatory

season (5.6 vs. 12.2, respectively; Palomino et al., 2014b) or anovulatory season (8.1 vs. 14.6, respectively; Palomino et al., 2013). Consistent with the premise that the stress of multiple handling events in a wild species is deleterious to a superovulatory response (Solti et al, 2000), we found that two doses of FSH given subcutaneously induced a greater ovarian response than twice daily intramuscular treatments in wood bison, and that a single subcutaneous dose of FSH resulted in a superovulatory response that did not differ from the two-dose regime (Toosi et al., 2013).

Although the ovulation-inducing effects of GnRH and pLH were not compared directly in our previous study in wood bison (Toosi et al., 2013), results of separate experiments reported therein provided rationale for the hypothesis that pLH was more effective than GnRH in inducing ovulation in superstimulated wood bison (i.e., mean of 8 vs. 3 CL per bison, respectively; Toosi et al., 2013). The use of hCG for inducing ovulation has been examined in superstimulated beef and dairy cattle (Madill et al., 1994; Baruselli et al., 2006; Bo et al., 2006), but no reports were found on the use of hCG in bison.

Wood bison are seasonal breeders with the ovulatory season between August and February and the anovulatory season between March and July (McCorkell et al., 2013a; Palomino et al., 2013; Palomino et al., 2014b). Superovulation during the anovulatory season has been reported in a number of seasonal species including sheep (Barret et al., 2004; Iida et al., 2003), goats (Baril et al., 1990), and wapiti (McCorkell et al., 2013b), but previous studies in bison were done only during the ovulatory season (Dorn et al., 1995; Toosi et al., 2013). Studies in cattle suggest that embryo development is affected by circulating concentrations of progesterone (Goto et al., 1987; Goto et al., 1988; Nasser et al., 2011), and since endogenous progesterone is low throughout the anovulatory season in wood bison (Matsuda et al., 1996), we speculated that

exogenous progesterone may be needed to obtain viable embryos in wood bison during the anovulatory season.

To improve the ovarian response and embryo production in wood bison, we evaluated novel superovulatory treatment protocols during the anovulatory and ovulatory seasons. During the anovulatory season (Experiment 1), the objectives were to determine the necessity of exogenous progesterone during ovarian superstimulation and compare the ovulation-inducing effects of pLH vs. hCG. During the ovulatory season (Experiment 2), the objectives were to compare the response to a single vs. split intramuscular dose of FSH in hyaluronan, and the ovulation-inducing effects of pLH vs. hCG.

5.3. Materials and methods

5.3.1. Animals

The experiments were done during the anovulatory season (May-June, Experiment 1) and the ovulatory season (September-October, Experiment 2) in the same year. Female wood bison (6 - 10 years old), with an average body condition score of 3.5 (scale of 1 to 5; Vervaecke et al., 2005), were maintained in pens (6-8 bison/pen) at the Native Hoofstock Centre, University of Saskatchewan. Animals were fed with alfalfa/grass hay and fresh water *ad libitum* and supplemented with approximately 1.5 kg/head/day of a mix of oats and barley. Protocols and use of bison were approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

5.3.2. Experiment 5.1 (anovulatory season)

A 2 x 2 factorial design was used to examine the effects of exogenous progesterone during ovarian superstimulation and the ovulatory response to pLH vs. hCG (Fig. 1). Emergence of a new follicular wave was induced in 20 female wood bison by ultrasound-guided aspiration of all follicles ≥ 5 mm in diameter (follicular ablation), as described previously (Bergfelt et al., 1997; Palomino et al., 2014a). On the day after follicular ablation (i.e., expected day of wave emergence; Day 0), the bison were given a single intramuscular dose of 400 mg of pFSH (Folltropin-V, Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada) diluted in 10 ml of 0.5% hyaluronan (5 mg/mL, MAP 5, Bioniche Animal Health). Half of the bison were given a progesterone-releasing intravaginal device (PRID, Vetoquinol Inc, Quebec, Canada) containing 1.55 g of progesterone in a stainless steel spiral covered with an inert silicone rubber matrix on Day 0. The PRID were removed on Day 4, and on Day 5, bison in each group (with or without progesterone) were divided into two subgroups and given either 25 mg of pLH (Lutropin-V, Bioniche Animal Health) or 3000 IU of hCG (Chorulon, Merck Animal Health, Kirkland, Quebec, Canada). The bison were artificially inseminated 12 and 24 hours later. Ova/embryos were collected by a nonsurgical technique 8 days after hCG treatment (Day 13; Fig. 1).

The ovarian response was recorded by carefully sketching the number, size and relative location of follicles ≥5 mm and CL during transrectal ultrasonography using a 7.5 MHz linear-array probe (ALOKA SSD-900, Tokyo, Japan), as described previously in wood bison (Palomino et al., 2014a). The ovaries were examined on Day 0, every 12 hours from Day 5 (12 hours after pLH or hCG treatment) to Day 8, and again at the time of ova/embryo collection on Day 13. Ovulation was defined as the disappearance of a follicle ≥9 mm from one examination to the next.

5.3.3. Experiment 5.2 (ovulatory season)

The 20 female wood bison used during the anovulatory season in Experiment 1 were used again during the ovulatory season in Experiment 2. At random stages of the estrous cycle, bison were given an intramuscular (i.m.) dose of prostaglandin (500 µg cloprostenol; Estrumate, Merck Animal Health) followed by transvaginal follicular ablation 8 days later to synchronize luteal and follicular development (Fig. 1). On the day following follicle ablation (expected day of follicular wave emergence, Day 0), bison were assigned randomly to two groups (n = 10/group) and given a total of 400 mg pFSH diluted in 0.5% hyaluronan as a single intramuscular dose (single dose group) or divided into 300 mg on Day 0 and 100 mg on Day 2 (split dose group). Prostaglandin (500 µg cloprostenol i.m.) was given on Day 3, ovulation-induction treatment with pLH (25 mg) or hCG (3000 IU) was given i.m. on Day 5, and artificial insemination was done 12 and 24 h later. Ovarian ultrasonography and ova/embryo collection were performed as described in Experiment 1 (Fig. 1).

5.3.4. Semen collection, handling, and artificial insemination

Chilled semen (5ºC) was used for artificial insemination in both experiments. For each experiment, semen was collected twice, three days apart, by electroejaculation of two mature wood bison bulls. The bulls were restrained without sedation in a hydraulic chute, as described previously (Lessard et al., 2009). After evacuating feces from the rectum and massaging the accessory sex glands and pelvic urethra for about 30 seconds, a probe with ventrally oriented electrodes (Pulsator IV, Lane Manufacturing, Denver, CO, USA) was introduced into the rectum and the tail was placed between the probe handles to maintain probe orientation and avoid

expulsion. Electrical stimulation was applied in a step-wise manner by rhythmically rotating the voltage control dial from zero to progressively higher levels at 3- to 4-second intervals at each power step (0 to 9) until ejaculation occurred (usually at power step 4). Semen was collected in a 15 ml tube attached to a plastic collection cone that was held over the tip of the extended penis with the use of a 20 cm long extension handle. Semen samples from the bulls were pooled and evaluated to determine volume, concentration, motility, and progressive motility. On average, collected semen had a total motility and progressive motility of 90% and 85%, respectively. These values did not differ between seasons. An egg-yolk-based extender (Triladyl, Minitube, Tiefenbach, Germany), was used to dilute the semen to a concentration of 300 x 10^6 sperm/ml and at at 5ºC. The pooled semen was placed in a 50 mL tube and the extender was added slowly at room temperature. The tube of diluted semen was placed in a beaker filled with room temperature water and left on the bench for 15 minutes. The beaker with the semen was then transferred to a cool room and maintained at 5ºC until use (≤60 hr). In both experiments, progressive motility after warming to 35ºC on the day of collection and 1 and 2 days later was, on average, 82%, 79%, and 76%, respectively.

For insemination, the superstimulated wood bison were restrained in a hydraulic chute without sedation (Palomino et al., 2014a). The chilled semen (at 5ºC) was loaded into 0.5 ml straws and placed in an artificial insemination gun. The gun was introduced through the cervix by transrectal manipulation and semen was deposited into the uterine body (total dose of 150 X 10^6 per insemination).

5.3.5. Embryo collection and evaluation

Non-surgical embryo collection was done using an interrupted-syringe method, as described in cattle (Mapletoft, 1986). The bison were restrained in a hydraulic chute without sedation and caudal epidural anesthesia was induced with 4 - 5 mL of 2% lidocaine hydrochloride (Bimeda-MTC, Animal Health Inc., Cambridge, ON, Canada; Palomino et al., 2014a). The tail was tied to the left and the vulva was carefully washed and disinfected using iodine detergent, water and isopropyl alcohol. The vulva was rinsed thoroughly with saline (0.9% sodium chloride, Hospira, Montreal, Quebec, Canada), and a 16 French silicone embryo collection catheter with a stainless steel stylet (Bioniche Animal Health) was passed through the vagina and cervix. The catheter was directed into one of the uterine horns near the uterine bifurcation and the stylet was removed in a step-wise manner to allow advancement of the catheter tip further into the uterine horn without damaging the endometrium. The balloon cuff was inflated with 5 to 8 mL of embryo collection medium at a site approximately half-way between the bifurcation and the tip of the uterine horn to fix the catheter in place and prevent reflux during ova/embryo collection. The stylet was then removed completely, and a catheter-tip syringe containing 20 – 30 mL of collection medium (Complete Flush, Bioniche Animal Health) was infused through the silicone catheter into the uterine lumen and aspirated during transrectal manipulation and agitation. Each uterine horn was flushed with 7 or 8 syringe volumes of medium. The contents of each syringe was deposited into a 75 micron filter (Emcon filter; Agtech, Manhattan, Kansas, USA) maintained at room temperature. After both uterine horns were flushed, the filter was taken to the lab for embryo searching. The contents of the filter were poured into a 90 mm Petri dish and the filter was rinsed using a commercial rinsing medium (Vigro Rinsing Solution, Bioniche Animal Health). Embryos were located and sorted by stereomicroscopy (SMZ 1000, Nikon Instrument Inc., Melville, NY, USA) at a magnification of 8X, and morphologically evaluated at a

magnification of 50X according to guidelines of the International Embryo Transfer Society (Stringfellow and Givens, 2010). Embryos classified as Grade 1 and 2 were considered transferable.

5.3.6 Statistical analyses

For both experiments, the number of follicles ≥9 mm on Day 5 was compared between superstimulation groups by t-test. The proportion of follicles ≥9 mm that ovulated per bison was compared by 2-way analysis of variance using a Generalized Linear Mixed Model. The number of CL on Day 13, number of ova and embryos collected, and timing of ovulation were compared among groups by two-way analysis of variance. Post hoc comparisons were made by Tukey's test. The proportion of bison that had <2 ovulations, was evaluated by logistic regression. The distribution of bison relative to the interval from first to last ovulation was compared by analysis of variance. An event with a probability of ≤0.05 of happening by chance alone was considered statistically significant. Data are presented as mean ± SEM, unless otherwise indicated.

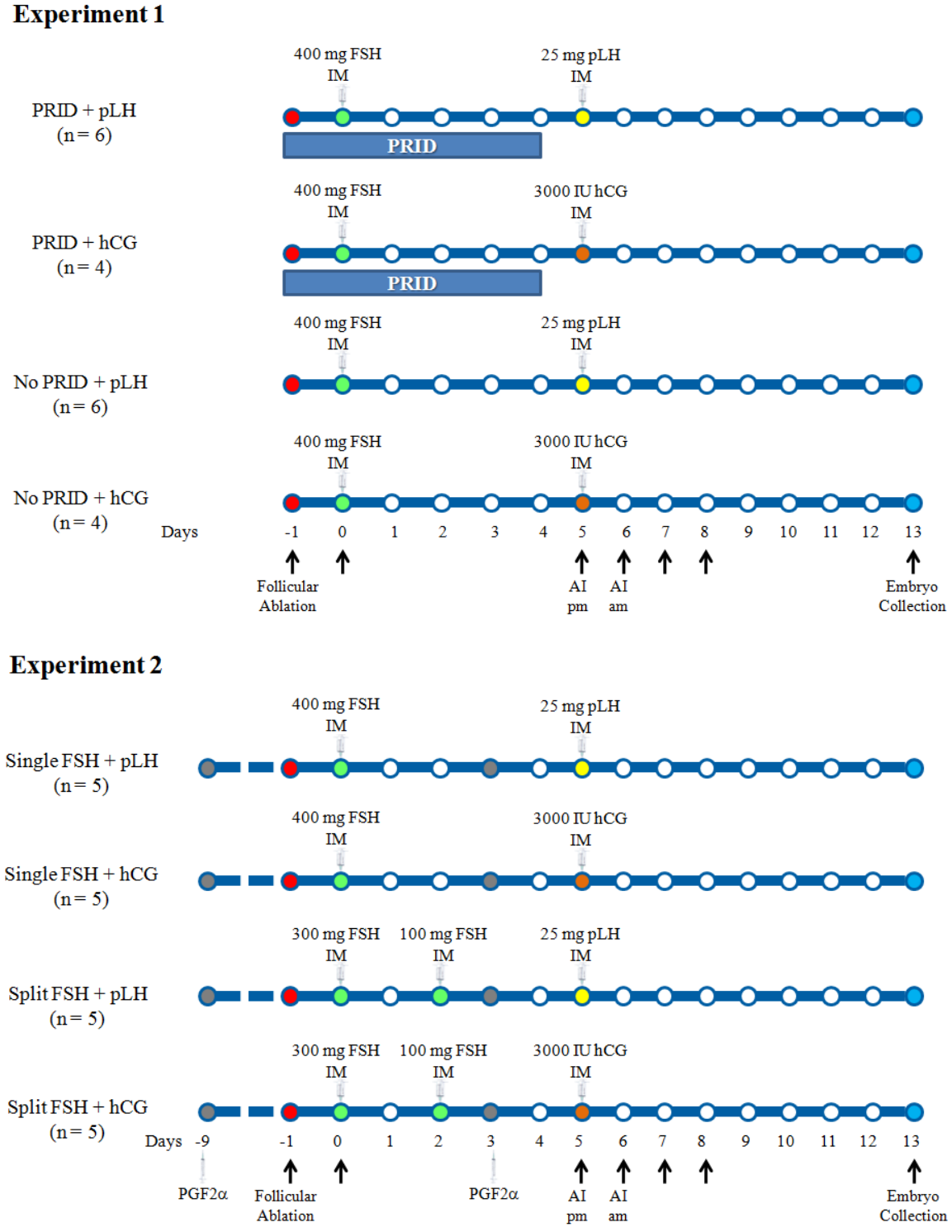

Fig. 5.1. Superstimulation protocols and treatment groups for Experiments 1 & 2 (n=number of wood bison per group). PRID: Progesterone-releasing intravaginal device. Follicular ablation (Day -1; red dot), FSH treatment (green dot) in single dose (Day 0) and split dose (Days 0 and 2) groups, PGF2α treatment (Days -9 and 3; gray dot), pLH treatment (Day 5; yellow dot), hCG treatment (Day 5; orange dot). AI: artificial insemination (Day 5 pm and Day 6 am). Arrows: ultrasound examinations, Embryo collection (Day 13; blue dot).

5.4. Results

5.4.1 Experiment 1 (anovulatory season)

There were no interactions between PRID treatments and ovulation inducing treatments (pLH vs hCG) for any of the end points (Table 5.1). There was no difference between progesterone groups in the number of follicles ≥9 mm on Day 5 (P = 0.66), but the proportion of follicles ≥9 mm on Day 5 that ovulated was greater in the hCG group than the pLH group (P ≤0.05), and tended to be greater in animals without a PRID (P = 0.10). The number of CL on Day 13 was greatest in bison treated with hCG and without PRID (P ≤0.05). The number of ova/embryos and transferable embryos did not differ among groups (Table 5.1).

Five of the 20 bison (25%) failed to ovulate at all. The proportion of bison that had <2 ovulations did not differ among groups (Table 5.2). The interval and the variation in the interval between ovulation-induction treatment (pLH or hCG) and the first ovulation were not different among groups. Within bison, the interval between the first and last ovulation was shorter in bison treated with a PRID than in those not treated with a PRID (P<0.05), but did not differ between pLH and hCG groups (P = 0.17; Table 5.2). The interval from first to last ovulation within bison ranged from 0 h (simultaneous) to 36 h; the interval was 12 h in most bison (45%, P<0.05; Fig. 5.2).

Table 5.1 Ovarian response & ova/embryo data (mean ±SEM) in wood bison (n) superstimulated with a single dose of FSH in 0.5% hyaluronan with or without a progesterone-releasing intravaginal device (PRID) and induced to ovulate with either pLH or hCG during the anovulatory season (Experiment 1).

End point*	**No PRID**	**PRID**	**Combined**
Follicles ≥ 9 mm on Day 5	8.5 ± 1.7 (n = 10)	7.7 ± 1.6 (n = 10)	8.1 ± 1.1 (n = 20)
Proportion of follicles that ovulated per bison (Number of ovulations / number of follicles ≥9 mm on Day 5)			
pLH	0.40 ± 0.13 (n = 6)	0.16 ± 0.08 (n = 6)	0.28 ± 0.08^{x} (n =12)
hCG	0.75 ± 0.09 (n = 4)	0.58 ± 0.21 (n = 4)	0.66 ± 0.11^{y} (n =8)
Combined	0.54 ± 0.10 (n = 10)	0.33 ± 0.11 (n = 10)	0.44 ± 0.08 (n = 20)
Number of corpora lutea per bison (Day 13)			
pLH	3.2 ± 0.9 (n = 6)	2.5 ± 1.5 (n = 6)	2.8 ± 0.8^{x} (n =12)
hCG	10.3 ± 1.9 (n = 4)	3.0 ± 1.6 (n = 4)	6.6 ± 1.8^{y} (n =8)
Combined	6.0 ± 1.5^{a} (n = 10)	2.7 ± 1.0^{b} (n = 10)	4.3 ± 0.9 (n = 20)
Number of ova/embryos per bison			
pLH	0.8 ± 0.5 (n = 6)	1.3 ± 1.0 (n = 6)	1.1 ± 0.5 (n =12)
hCG	2.5 ± 1. 9 (n = 4)	0.8 ± 0.8 (n = 4)	1.6 ± 1.0 (n = 8)
Combined	1.5 ± 0.8 (n = 10)	1.1 ± 0.6 (n =10)	1.3 ± 0.5 (n = 20)
Number of transferable embryos per bison			
pLH	0.3 ± 0.2 (n = 6)	0.7 ± 0.7 (n = 6)	0.5 ± 0.3 (n =12)
hCG	0.8 ± 0.5 (n = 4)	0.5 ± 0.5 (n = 4)	0.6 ± 0.3 (n = 8)
Combined	0.5 ± 0.2 (n =10)	0.6 ± 0.4 (n =10)	0.6 ± 0.2 (n = 20)

*No interaction between main effects for any endpoint

[ab] Within rows, values with different superscripts are different ($P<0.05$)

[xy] Within columns, values with different superscripts are different ($P<0.05$)

Table 5.2 Timing of ovulation (hours, mean ±SEM) in wood bison (n) superstimulated with a single dose of FSH in 0.5% hyaluronan with or without a progesterone-releasing intravaginal device (PRID) and induced to ovulate with either pLH or hCG during the anovulatory season (Experiment 1).

End point*	**No PRID**	**PRID**	**Combined**
Proportion of bison that had <2 ovulations			
pLH	2/6 (33%)	3/6 (50%)	5/12 (42%)
hCG	0/4 (0%)	2/4 (50%)	2/8 (25%)
Combined	2/10 (20%)	5/10 (50%)	7/20 (35%)
Interval from pLH or hCG treatment to first ovulation (hours)[2]			
pLH	28.8 ± 2.9 (n = 5)	28.0 ± 3.1 (n = 3)	28.5 ± 2.2 (n = 8)
hCG	24.0 ± 0.0 (n = 4)	28.0 ± 4.0 (n = 3)	25.7 ± 1.7 (n = 7)
Combined	26.7 ± 1.8 (n = 9)	28.0 ± 2.5 (n = 6)	27.2 ± 1.4 (n = 15)
Variation in interval from pLH or hCG treatment to first ovulation among bison (Residuals)[3]			
pLH	5.8 ± 0.6 (n = 5)	5.3 ± 1.0 (n = 3)	5.6 ± 0.6 (n = 8)
hCG	0.0 ± 0.0 (n = 4)	11.0 ± 6.6 (n = 3)	5.5 ± 3.6 (n = 7)
Combined	3.2 ± 2.2 (n = 9)	8.6 ± 3.6 (n = 6)	5.6 ± 1.7 (n = 20)
Interval from first ovulation to last ovulation within bison (hours)[4]			
pLH	12.8 ± 2.9 (n = 5)	8.0 ± 0.0 (n = 3)	11.0 ± 2.0 (n = 8)
hCG	27.0 ± 9.0 (n = 4)	8.0 ± 0.0 (n = 3)	18.9 ± 6.2 (n = 7)
Combined	19.1 ± 4.0[a] (n = 9)	8.0 ± 0.0[b] (n = 6)	14.7 ± 3.1 (n = 15)

*No interaction between main effects for any endpoint

[ab] Within rows, values with different superscripts are different (P<0.05)

[xy] Within columns, values with different superscripts are different (P<0.05)

Table 5.3. Ovarian response and ova/embryo data (mean ±SEM) in wood bison (n) superstimulated with a single vs. split dose of FSH in 0.5% hyaluronan and induced to ovulate with either pLH or hCG during the ovulatory season (Experiment 2).

End point*	**Single FSH**	**Split FSH**	**Combined**
Follicles ≥9 mm on Day 5	5.9 ± 1.1[a] (n = 10)	10.5 ± 1.4[b] (n = 10)	8.2 ± 1.0 (n = 20)
Proportion of follicles that ovulated per bison (Number of ovulations / number of follicles ≥9 mm on Day 5)			
pLH	0.41 ± 0.13 (n = 5)	0.61 ± 0.16 (n = 5)	0.51 ± 0.10[x] (n =10)
hCG	0.66 ± 0.05 (n = 5)	0.81 ± 0.12 (n = 5)	0.74 ± 0.07[y] (n =10)
Combined	0.54 ± 0.08 (n = 10)	0.71 ± 0.10 (n = 10)	0.62 ± 0.06 (n = 20)
Number of corpora lutea per bison on Day 13			
pLH	1.4 ± 0.4 (n = 5)	6.2 ± 1.7 (n = 5)	3.8 ± 1.2[x] (n = 10)
hCG	4.6 ± 1.2 (n = 5)	8.0 ± 0.6 (n = 5)	6.3 ± 0.8[y] (n = 10)
Combined	3.0 ± 0.8 [a] (n = 10)	7.1 ± 0.9 [b] (n = 10)	5.1 ± 0.8 (n = 20)
Number of ova/embryos per bison			
pLH	0.0 ± 0.0 (n = 5)	2.3 ± 0.9 (n = 5)	1.3 ± 0.6 (n = 10)
hCG	1.6 ± 0.9 (n = 5)	2.2 ± 1.0 (n = 5)	1.9 ± 0.6 (n = 10)
Combined	1.0 ± 0.5 (n = 10)	2.2 ± 0.6 (n = 10)	1.6 ± 0.4 (n = 20)
Number of transferable embryos per bison			
pLH	0.0 ± 0.0 (n = 5)	0.8 ± 0.4 (n = 5)	0.4 ± 0.3 (n = 10)
hCG	1.2 ± 0.7 (n = 5)	0.4 ± 0.4 (n = 5)	0.8 ± 0.4 (n = 10)
Combined	0.8 ± 0.4 (n = 10)	0.6 ± 0.3 (n = 10)	0.7 ± 0.2 (n = 20)

*****No interaction between main effects for any endpoint

[ab] Within rows, values with different superscripts are different ($P<0.05$)

[xy] Within columns, values with different superscripts are different ($P<0.05$)

Table 5.4 Timing of ovulation (hours, mean ±SEM) in wood bison (n) superstimulated with a single vs. split dose of FSH in 0.5% hyaluronan and induced to ovulate with either pLH or hCG during the ovulatory season (Experiment 2).

End point*	**Single FSH**	**Split FSH**	**Combined**
Proportion of bison that had <2 ovulations			
pLH	2/5 (40%)	1/5 (20%)	3/10 (30%)
hCG	0/5 (0%)	0/5 (0%)	0/10 (0%)
Combined	2/10 (20%)	1/10 (10%)	3/20 (15%)
Interval from pLH or hCG treatment to first ovulation (hours)[2]			
pLH	33.0 ± 3.0 (n = 4)	33.0 ± 3.0 (n = 4)	33.0 ± 2.0 (n = 8)
hCG	33.6 ± 2.4 (n = 5)	28.8 ± 2.9 (n = 5)	31.3 ± 2.0 (n = 10)
Combined	33.3 ± 1.8 (n = 9)	30.7 ± 2.1 (n = 9)	32.0 ± 1.4 (n = 18)
Variation in interval from induce-ovulation treatment to first ovulation among bison (Residuals)[3]			
pLH	4.5 ± 1.5 (n = 4)	4.5 ± 1.5 (n = 4)	4.5 ± 1.0 (n = 8)
hCG	3.8 ± 1.4 (n = 5)	5.8 ± 0.6 (n = 5)	4.8 ± 0.8 (n = 10)
Combined	4.1 ± 1.0 (n = 9)	5.2 ± 0.7 (n = 9)	4.7 ± 0.6 (n = 18)
Interval from first ovulation to last ovulation within bison (hours)[4]			
pLH	6.0 ± 3.0 (n = 4)	12.0 ± 3.0 (n = 4)	9.0 ± 2.3[x] (n = 8)
hCG	14.0 ± 3.8 (n = 5)	19 ± 3.8 (n = 5)	16.5 ± 2.7[y] (n = 10)
Combined	10.4 ± 2.7 (n = 9)	15.9 ± 2.6 (n = 9)	13.2 ± 2.0 (n = 18)

*****No interaction between main effects for any endpoint

[xy] Within columns, values with different superscripts are different (P<0.05)

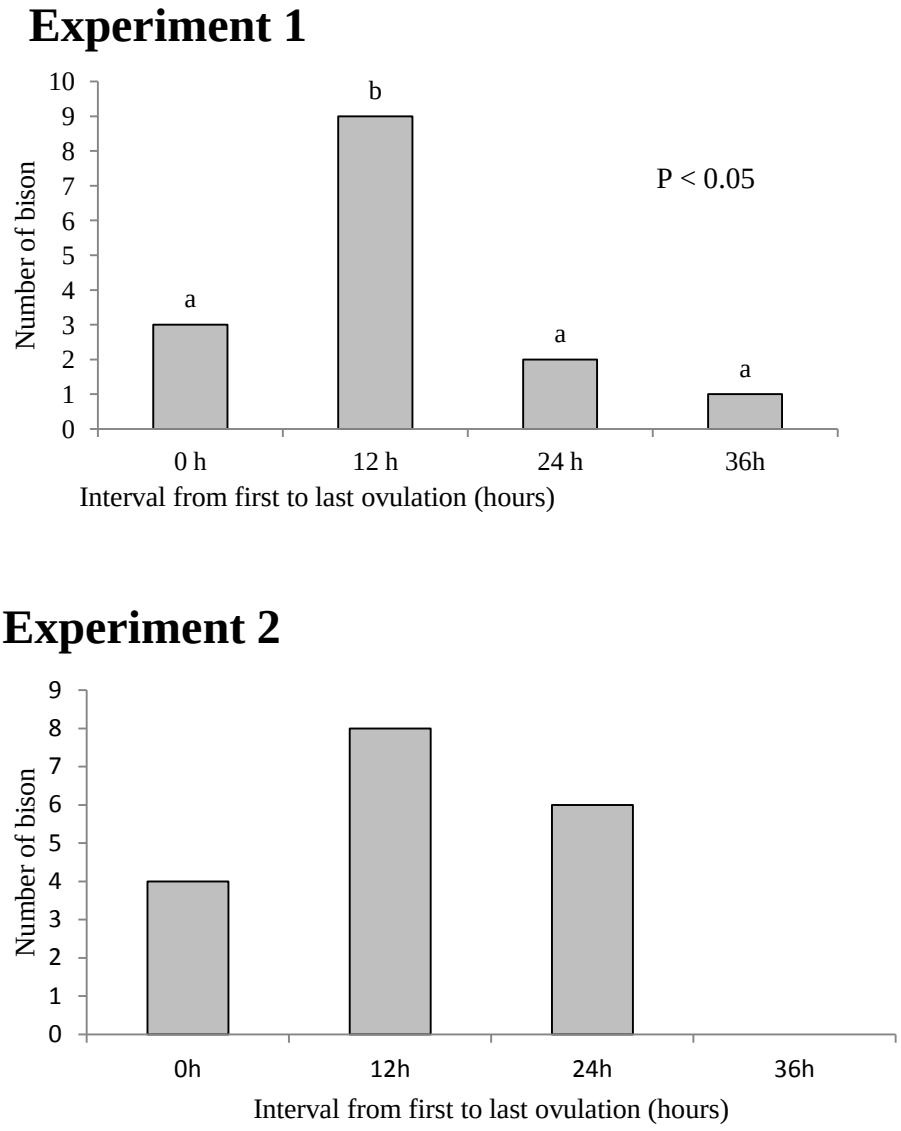

Fig. 5.2 Occurrence of ovulation dispersed over several hours in Experiment 1 (Above) and Experiment 2 (Below). 0h: Number of bison that ovulated at once. 12h, 24h, 36h: Number of bison that ovulate over a period of 12, 24, and 36 hours respectively.

5.4.2 Experiment 2 (ovulatory season)

There were no interactions between the number of FSH treatments (single versus split) and ovulation inducing treatments (pLH vs. hCG; Table 5.3). The number of follicles ≥9 mm on Day 5 was greater in the split dose group than in the single dose group ($P < 0.05$). The proportion of

follicles ≥9 mm on Day 5 that ovulated was greater in bison treated with hCG than pLH (P ≤0.05), and tended to be greater in the split dose than single dose FSH group (P = 0.10). The number of CL was greatest in bison treated with a split dose of FSH and hCG (P ≤0.05). The number of ova/embryos and transferable embryos did not differ among groups.

Two bison (10%) failed to ovulate, one given a single dose of FSH and pLH and the other given a split dose of FSH and pLH. All bison treated with hCG ovulated. The proportion of bison that had <2 ovulations was not different among groups (Table 5.4). The interval and the variation in the interval between ovulation-induction treatment (pLH or hCG) and the first ovulation did not differ among groups. Within bison, the interval between the first and last ovulation did not differ between single dose vs. split dose FSH groups (P = 0.36), but was shorter in the pLH vs. hCG group (P ≤0.05; Table 5.4). The interval from first to last ovulation within bison ranged from 0 h (simultaneous) to 24 h; the interval was 12 h in most bison (40%, Fig. 5.2).

5.5. Discussion

Overall, the ovulatory response to hCG was greater than pLH in superstimulated wood bison; i.e., 6.6 vs 2.8 CL during the anovulatory season and 6.3 vs 3.8 CL during the ovulatory season. These results were surprising as we expected similar results since both gonadotropins act directly at an ovarian level (Ascoli et al., 2002). However, some structural differences may explain why we obtained dissimilar results. The gonadotropins have identical α subunits but differing β subunits (Pierce and Parsons, 1981). The presence of a carboxyl terminal peptide and the type and amount of glycosylation are the most different features of the β subunit and influence the circulating half life (Casarini et al., 2012; Matzuk et al., 1990). The half-life of hCG has been reported to be about 30 hours (Rizkallah et al., 1969, Schmitt et al., 1996),

whereas the half-life of the endogenous LH is about 60 minutes (Robertson et al., 1991). A longer period of ligand availability and occupancy on the follicular cell receptors as a result of the longer β chain may explain the greater ovulatory response to hCG than LH in the present study. In addition, a longer period of availability may have allowed sufficient time and stimulus for follicles that were immature at the time of treatment to mature and gain ovulatory capacity. The latter is consistent with greater asynchrony in ovulations within bison (longer interval from first to last ovulation) in the hCG group; i.e., larger, more mature follicles at the time of treatment may have ovulated sooner than the smaller, immature follicles (Adams et al., 1993). The use of hCG for inducing ovulation in superstimulated bison has not been reported previously, but has been reported for domestic ruminants (Madill et al., 1994; Wani et al., 1997) and horses (Niswender et al., 2003), in which the ovarian response did not differ from that of GnRH or pLH treatment.

In the present study, ovarian superstimulation was achieved in wood bison following the use of simplified FSH treatment protocols during both the anovulatory and ovulatory seasons. A conventional scheme of ovarian superstimulation involves intramuscular administration of FSH twice a day for four or five days (Mapletoft et al., 2002). Twice daily treatment has been considered necessary because the circulating half-life of FSH in the cow is only about 5 hours (Laster, 1972). However, handling stress was considered a major factor adversely affecting results in early attempts to superstimulate bison (Dorn, 1995), so we tested simplified protocols that would involve fewer animal handlings (Toosi et al., 2013; present study), similar to those reported recently in cattle (Tribulo et al., 2012). In the present study, the number of CL was more than two-fold greater in bison given a split dose of FSH vs. a single dose (7.1 vs. 3.0). This is consistent with recent studies with 1% hyaluronan in cattle (Tribulo et al., 2011; 2012), but

contrasts with results of our previous study in wood bison (Toosi et al., 2013) in which no difference was detected in the superovulatory response between single dose (FSH in 1% hyaluronan) vs split dose (FSH in saline given subcutaneously). The disparity between studies may be attributed to a difference in the concentration of hyaluronan used to dilute the FSH preparation in the present study (0.5%) vs. Toosi's study (1%). It is noteworthy that 2% hyaluronan was effective for superovulation as a single treatment in cattle, but difficulties in mixing FSH with 2% hyaluronan which was very viscous (Tribulo et al., 2011) led to a split-dose protocol using 1.0 or 0.5% hyaluronan which could be mixed with FSH with little difficulty (Tribulo et al., 2012). The superovulatory response to the split dose of FSH in the present study (7.1 CL/bison) was similar to that of our previous study in wood bison treated with FSH twice daily for 4 days (7.3 CL per bison), but greater than in an early study in plains bison in which FSH was given twice daily (3.6 CL/bison; Dorn et al., 1990). In addition to the effect of handling stress, the lower response in the latter study may be related to treatment being initiated without regard to follicular wave emergence; i.e., follicular wave emergence was not synchronized prior to treatment (Nasser et al., 1993; Adams et al., 1993; 1994).

The rationale for examining the effect of progesterone during the anovulatory period in the present study was based on the potential adverse effect of an extended period of low progesterone on oocyte competence, on the endometrium, and early embryo development (Lonergan, 2011). In Experiment 1 of the present study, circulating progesterone concentrations were minimal for >2 months prior to superstimulatory treatment (Vervaecke and Schwarzenberger, 1996; Palomino et al, 2014a; McCorkell et al., 2013a), so the inclusion of a progesterone-treated group permitted a test of the effect of progesterone on the ovulatory response and ova/embryo production. As a result of a greater proportion of the large follicles that

ovulated, the superovulatory response was greater in bison not given a PRID during superstimulation, but the number of ova/embryos and freezable embryos (IETS grades 1 and 2) collected did not differ between the PRID and no PRID groups. This is consistent with results in cattle comparing Wave 1 (low progesterone) vs. Wave 2 (high progesterone; Adams et al., 1994a); however, we interpret these results with caution because of the low number of embryos collected in Experiment 1. In this regard, an artificially extended period of low progesterone in Nelore cattle did not affect the superovulatory response but was associated with the collection of a greater proportion of unfertilized ova (Nasser et al., 2011). Further studies are needed to determine the effect of progesterone on embryo quality during the anovulatory season in wood bison.

In total, 11 transferable embryos were collected from 20 female wood bison during the anovulatory season and 11 transferable embryos were collected from same females during the ovulatory season. Our first experience collecting embryos from superovulated wood bison (Toosi et al., 2013) was done during the ovulatory season, and 5 transferable embryos were collected from 20 females. Despite a 2-fold increased in the number of freezable embryos in the present study, our results (mean of 0.7 embryos) remains well below the number of transferable embryos collected from cattle using a split dose of FSH (3.7 embryos; Tribulo et al., 2012) and from the world average in cattle (6.8 transferable embryos; Perry, 2014). However, this is the first report of the recovery of ova/embryos from superstimulated wood bison during the anovulatory season and the response was similar to that of the ovulatory season. If the embryo recovery rate can be improved commensurate with the ovulation rate (average over both seasons: 1.4 ova-embryos, 0.6 freezable embryos, and 4.3 CL per bison; ratios: 0.31 and 0.14, respectively) this technology may be used effectively for embryo production throughout the year. By comparison, the ratio of

ova-embryos and transferrable embryos collected relative to the number of CL in superstimulated cattle is over two-fold higher (approximately 0.69 and 0.40, respectively; Adams et al., 1994a; Nasser, 2011). The ratio is similarly low in superstimulated water buffalo (*Bubalus bubalis*; 2.2 ova-embryos and 5.7 CL; ratio: 0.39; Baruselli et al., 1999). The reason for the discrepancy between the number of CL and the number of ova/embryos recovered requires further investigation.

The interval from treatment to ovulation and interval between first and last ovulations within animal is important to determine the optimum timing of insemination in a superovulatory protocol. In the present study, the interval from pLH or hCG treatment to first ovulation was similar between seasons (27-32 hours), and was similar to that reported in superstimulated cattle after GnRH or pLH treatment (29 - 31 hours; Bo et al., 2006). The interval from the first to the last ovulation within bison was also similar between seasons (13-15 hours), and was comparable or slightly longer than that reported in cattle (12 to 14 hours; Yadav et al., 1986; 1-6 hours, Adams et al., 1994a; 4 - 14 hours, Purwantara et al., 1994; 8 - 54 hours, Bo et al., 2006). Because of the extended period of time over which ovulations took place, the second insemination in the present experiment may have improved fertilization rate in wood bison, as reported in cattle (Donaldson, 1985).

In summary, treatment with hCG resulted in a greater ovulatory response than pLH following superstimulation of wood bison, and treatment with a split-dose FSH protocol resulted in a greater ovarian superstimulatory response than a single dose protocol. Exogenous progesterone had no effect on the ovarian response or embryo production during the anovulatory season. The ovarian response (4.7 CL per bison) and number of ova/embryo (1.4 per bison) was similar between anovulatory and ovulatory seasons, but the reason for the wide disparity between

ovarian response and embryo collection rate remains unknown. The timing of ovulation and variation in the interval from first to last ovulation within bison will be useful for adjustment in the timing and number of inseminations in future studies to improve embryo production.

5.6. Acknowledgements

The authors thank Dr. Behzad Toosi and Dr. Nick Hawkins for help with data collection. We also thank Bioniche Animal Health Canada Inc. for providing Folltropin-V, Lutropin-V, MAP-5 and supplies for the embryo recovery, and Merck Animal Health for providing Chorulon, and Minitube of America for providing Triladyl for diluting the semen.

CHAPTER 6.

DOES THE ADDITION OF eCG AND PROGESTERONE INFLUENCE OVARIAN RESPONSE AND EMBRYO PRODUCTION IN SUPERSTIMULATED WOOD BISON (BISON BISON ATHABASCAE) DURING THE ANOVULATORY AND OVULATORY SEASONS?

Relationship of this study to the dissertation:

In Chapter 5, we found a greater ovarian response in wood bison superstimulated with two doses of FSH in hyaluronan and induced to ovulate with a single dose of hCG. Additionally, no effect of exogenous progesterone on superovulatory response and embryo quality was found. However, embryo quality results should be taken with caution due to the low number of embryos collected. Therefore, we believed that if ovarian response could be increased, a greater pool of embryo would be available to reliably evaluate embryo quality,. In Chapter 6, we investigated the effect of the addition of a low dose of eCG on superovulatory response during the ovulatory and anovulatory seasons and whether the addition of exogenous progesterone during the anovulatory season would improve fertilization rates and embryo quality. Results of Chapter 6 could lead to the conclusion that exogenous progesterone should be used in wood bison if transferable embryos are to be produced throughout the year.

6.1 Abstract

Experiments were done to determine if a low dose of eCG at the end of the superstimulation treatment protocol improves the ovarian response and embryo production in superstimulated wood bison during both anovulatory (Experiment 1) and ovulatory (Experiment 2) seasons, and if exogenous progesterone improves fertilization rates and embryo quality during the anovulatory season. The bison were synchronized by follicular ablation (Day -1) and given 400 mg of FSH (diluted in 0.5% hyaluronan) in split doses of 300 mg on Day 0 and 100 mg on Day 2. In Experiment 1 (anovulatory season), bison (n-26) were assigned randomly to 3 groups wherein superstimulatory treatment was accompanied by exogenous progesterone alone, eCG alone, or progesterone + eCG. A progesterone-releasing intravaginal device (PRID) was inserted on Day 0 and removed on Day 4 in the progesterone-treated groups. In the eCG-treated groups, a single dose of 400 IU of eCG was given i.m. on Day 3. A single dose of 3000 IU of hCG was given i.m. on Day 5 and bison were inseminated 12 and 24 h later. Ova/embryos were collected 8 days after hCG treatment. In Experiment 2 (ovulatory season), bison (n = 24) were assigned to two groups in which superstimulatory treatment with FSH as in Experiment 1 with or without eCG was administered with no progesterone supplementation. Corpus luteum function was controlled by treatment with PGF_{2a} 8 days before follicle ablation and on Day 3 of FSH treatment. Treatment with eCG and hCG, as well as insemination and ova/embryo collection were as described for Experiment 1. In Experiment 1, although the number of follicles that ovulated was lowest in bison treated with progesterone + eCG ($P < 0.05$), there were no differences among groups in the number of CL at the time of embryo collection, number of ova/embryos, or the number of freezable embryos. In Experiment 2, no difference between groups (eCG vs. no eCG) was detected for any end-point. Data in each experiment were

combined among groups to compare the effect of season (anovulatory vs ovulatory). The number of follicles ≥9 mm on Day 5 and the number of CL were greater ($P < 0.05$) during the anovulatory season, but the number of freezable embryos tended to be greater ($P = 0.06$) during the ovulatory season. In conclusion, the ovarian superovulatory response was not improved by the addition of eCG at the end of the superstimulation treatment protocol in wood bison and exogenous progesterone had no effect on the ovulatory response or embryo quality during the anovulatory season. The apparent effect of season on embryo production is worthy of further investigation.

6.2. Introduction

Bison herds in Wood Buffalo National Park (WBNP) in northern Alberta, Canada, are the most genetically diverse in the world (Wilson et al., 2005). However, wood bison herds in and around the park are infected with brucellosis and tuberculosis (Joly and Messier, 2001; 2004b; 2005). The interaction between the effects of these cattle-derived diseases and predation may account for the decline and stasis of the wood bison population since the early 1970s (Jolie and Messier, 2004a). Further decline in the population jeopardizes the genetic diversity and long-term viability of wood bison (Wilson et al., 2005; McFarlane et al., 2006; COSEWIC 2013). Therefore, reproductive technologies (i.e., superovulation and embryo transfer) are being developed in wood bison in an effort to preserve their genetic diversity (Toosi at al., 2013; Palomino Chapter 4; Chapter 5).

Embryos produced through superovulation may be conserved in germplasm biobanks to provide a high level of insurance against the loss of genetic diversity (Wild, 1992; Holt et al., 1996; Solti et al., 2000). Conventional protocols for superovulation in cattle include

administration of FSH twice daily for 4 to 5 days (i.e., eight treatments; Mapletoft et al., 2002). The lack of success in early superovulatory attempts in bison was attributed to the stress of multiple handling required for FSH treatments (Dorn et al., 1995; Othen et al., 1999). However, superstimulatory treatments in these early studies were initiated without regard to follicular wave status, which may also have contributed to the poor response in bison. By avoiding the suppressive effect of the dominant follicle on subordinate follicles within a follicular wave (Adams et al., 1993), the ovarian superstimulatory response in cattle is higher when treatment is initiated near the time of follicular wave emergence (Nasser et al., 1993; Adams et al., 1994a). In this regard, induction of new wave emergence has enabled self-appointed scheduling of superstimulatory treatment to optimize the ovarian response in cattle (Bo et al., 2008).

In a recent series of studies designed to test the efficacy of less frequent superstimulatory treatment protocols in wood bison, significantly more ovulations were induced using a 2-dose vs. 4-dose protocol, and the response to a single dose of FSH diluted in 1% hyaluronan was not different from a 2-dose protocol (Toosi et al., 2013). However, the ovarian response was greater in wood bison given a 2-dose versus single dose protocol using FSH diluted in 0.5% hyaluronan (Palomino Chapter 5). Despite improvement in the superovulatory response in wood bison, the embryo collection rate remains less than 1 transferable embryo per bison, regardless of season (ovulatory or anovulatory; Palomino Chapter 5). The addition of low doses of eCG to conventional FSH superovulatory protocols has been used in *Bos indicus* cattle to increase the ovulatory response and embryo collection rate (Mattos et al., 2011). Equine chorionic gonadotropin is a glycoprotein secreted by the endometrial cups of the equine placenta (Allen and Moor, 1972). It has both FSH- and LH-like effects and has a long circulating half-life of 3 to 5 days in cattle (Murphy and Martinuk, 1991). The provision of extra LH-like activity may

promote maturation and ovulatory capacity of multiple follicles induced by superstimulatory treatment.

Studies regarding the requirement for progesterone prior to or during superstimulatory treatment have been contradictory. Early studies in cattle suggested that circulating concentrations of progesterone at the time of the FSH treatment was positively related to the superovulatory response and number of transferable embryos (Goto et al., 1987; Goto et al., 1988), but treatments were initiated without regard to follicular wave status. In a later study, the ovarian response and embryo collection rate did not differ when the FSH treatment was initiated at the beginning of the first follicular wave (low progesterone) or second follicular wave (high progesterone) of the estrus cycle in cattle (Adams et al., 1994a). Recently, however, a greater number of embryos were collected from superstimulated Nelore cows treated with exogenous progesterone vs no progesterone after synchronization of follicular wave emergence (Nasser et al., 2011). In wood bison, circulating concentrations of progesterone remain low (approximately 1 ng/mg) throughout the anovulatory season (Matsuda et al., 1996). Thus, we speculate that exogenous progesterone may be needed during the anovulatory season to optimize production of of viable embryos in wood bison.

The objectives of the present study were to determine if a low dose of eCG at the end of the superstimulation protocol improves the ovarian response and embryo production rate in superstimulated wood bison during ovulatory and anovulatory seasons, and if exogenous progesterone improves embryo production during the anovulatory season. In addition, the study provided the opportunity to examine the effect of season on the superovulatory response and embryo collection rate in wood bison.

6.3. Materials and methods

6.3.1. Facility and bison

The experiments were performed at the Native Hoofstock Centre, University of Saskatchewan (52°08'N, 106°38'W), Saskatoon, Canada, during the anovulatory season (Experiment 1) and the ovulatory season (Experiment 2). Wood bison (6 - 10 years old) with an average body condition score of 3.5 (scale of 1 to 5; Vervaecke et al., 2005) were used in this study. The bison had free access to supplemental hay and fresh water, and were handled according to protocols approved by the University of Saskatchewan's Animal Research Ethics Board.

6.3.2. Experiment 1 (anovulatory season, May-June)

To determine the effects of eCG and exogenous progesterone during ovarian superstimulation, wood bison (n=26) were assigned randomly to three groups in which ovarian superstimulatory treatment was augmented by i) progesterone (n=8), ii) eCG (n=9), or iii) progesterone and eCG (n=9; Fig. 6.1). Before ovarian superstimulation, follicular wave emergence (Day 0) was induced by ultrasound-guided aspiration of follicles ≥5 mm in diameter (follicular ablation, Day -1), as described previously (Palomino et al., 2014a). Ovarian superstimulation was induced with pFSH (400 mg Folltropin-V diluted in 10 ml of 5 mg/ml of hyaluronan, Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada) given intramuscularly on Day 0 (300 mg) and Day 2 (100 mg). A progesterone-releasing intravaginal device (PRID, Vetoquinol Inc, City, Quebec, Canada) was inserted in the vagina on the day of follicular ablation in the progesterone-treated groups. On Day 3, the eCG-treated groups were given 400 IU of eCG intramuscularly. On Day 4, the PRID was removed from respective groups

and on Day 5 bison in all groups were given 3000 IU of hCG (Chorulon, Merck Animal Health, Kirkland, Quebec, Canada) intramuscularly. The bison were artificially inseminated with chilled semen (5ºC) 12 and 24 hours after hCG treatment. Embryos were collected non-surgically 8 days after hCG treatment (Fig. 6.1).

Ovarian status and response were evaluated by transrectal ultrasonography using a 7.5 MHz linear-array transducer (MyLab Five, Esaote North America, Inc., Indianapolis, IN, USA) as previously described in wood bison (McCorkell et al., 2013a; Palomino et al., 2014a). The ovaries were examined on Day 0 to detect wave emergence, on Days 5, 6, and 7 to detect ovulation, and on Day 13 to record the number of CL. During each examination, a sketch of the ovaries was made to record the number, size, and relative position of follicles ≥3 mm and CL. Ovulation was defined as the disappearance of a follicle ≥9 mm from one examination to the next.

6.3.3. Experiment 2 (ovulatory season, September-October)

Wood bison (n=24) were assigned randomly to two groups in which ovarian superstimulatory treatment was augmented or not augmented with eCG (n=12/group; Fig. 1). Ovarian synchrony was induced among bison by giving a luteolytic dose of prostaglandin (500 µg Cloprostenol im, Estrumate, Merck Animal Health, Kirkland, Quebec, Canada) followed 8 days later by follicular ablation (Day -1), as described in Experiment 1. The bison in both groups were given FSH (Folltropin diluted in 10 mL of 5 mg/mL hyaluronan, Bioniche Animal Health Canada) intramuscularly on Day 0 (300 mg) and Day 2 (100 mg). On Day 3, a single dose of 400 IU eCG (Pregnecol, Bioniche Animal Health Canada Inc., Belville, Ontario, Canada) was given intramuscularly to the eCG group, and luteolytic dose of prostaglandin was given to both groups. On Day 5, bison in both groups were given 3000 IU hCG intramuscularly (Chorulon, Merck

Animal Health, Kirkland, Quebec, Canada). Transrectal ultrasonography, artificial insemination, and embryo collection were done as described in Experiment 1 (Fig. 1).

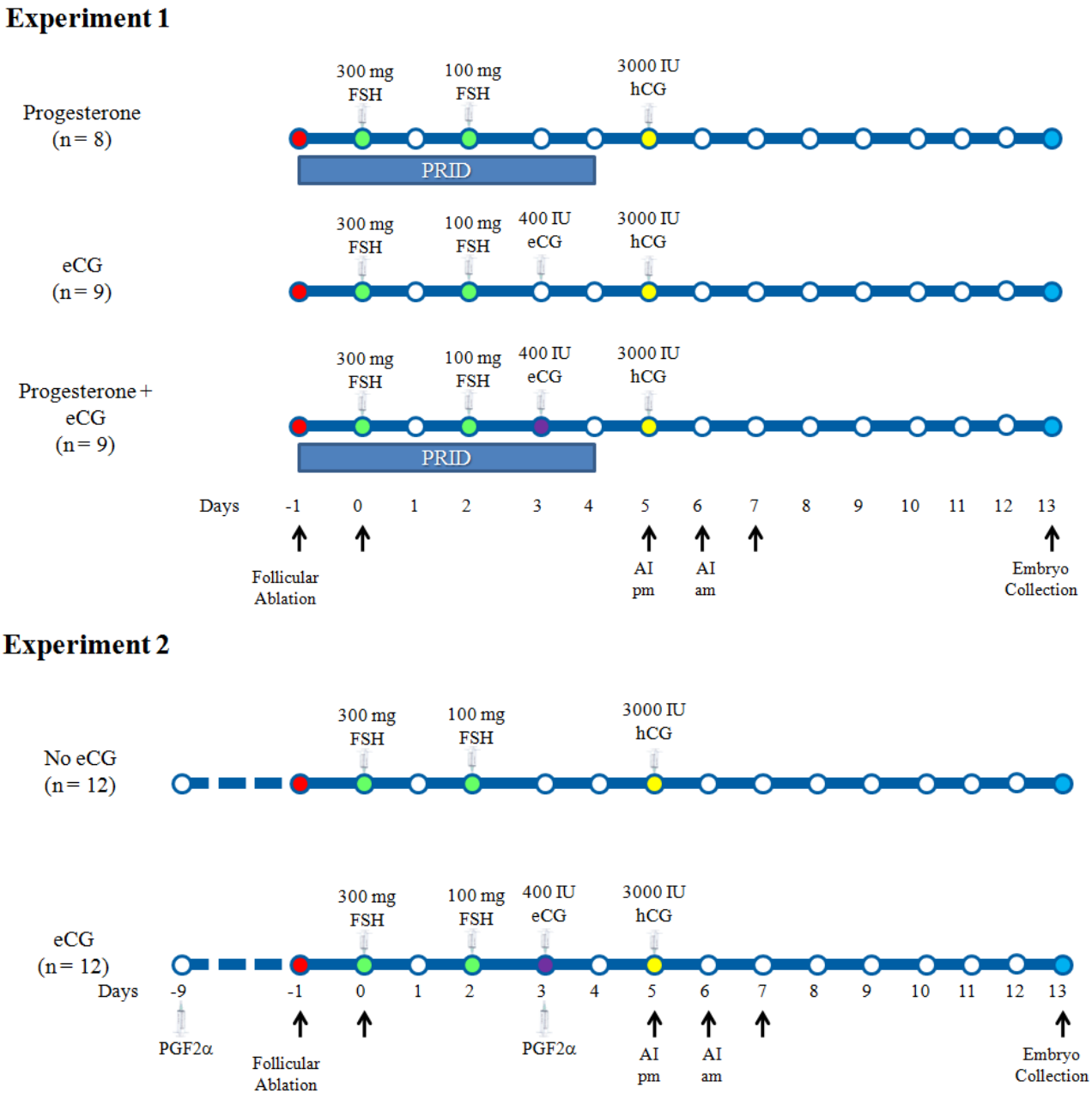

Fig. 1. Superovulatory treatment and examination protocols for Experiment 1 (anovulatory season) and Experiment 2 (ovulatory season) in wood bison. PRID: Progesterone-releasing intravaginal device. Follicular ablation (red dots), FSH treatment (green dots), eCG treatment (purple dots), hCG treatment (yellow dots), embryo collection (blue dots). PGF2α administration (Days -9 and 3), AI: Artificial insemination. Arrows: days of ovarian examination.

6.3.4 Semen handling and artificial insemination

Wood bison semen was collected by electroejaculation without sedation, as reported previously (Palomino Chapter 5). Ejaculates collected from two bulls were pooled and evaluated to determine volume, concentration, motility, and progressive motility. Pooled semen was diluted in egg-yolk-based extender Triladyl (Minitube, Tiefenbach, Germany) to achieve a concentration of 300 x 10^6 sperm/ml. Diluted semen was preserved at 5^{o}C in a cool room for up to 60 hr. Motility and progressive motility after warming was not less than 80% and 75% respectively on each day of insemination. For insemination, the superstimulated wood bison were restrained in a hydraulic chute without sedation (Palomino et al., 2014a). The chilled semen was loaded into 0.5 ml straws (insemination dose, 150 X 10^6 sperm) and placed in an artificial insemination gun. The gun was introduced through the cervix by transrectal manipulation and semen was deposited into the uterine body.

6.3.5. Embryo collection and evaluation

The interrupted-syringe method was used to collect embryos from superovulated female wood bison as previously described (Palomino Chapter 5). Epidural anesthesia was induced with 4 - 5 mL of 2% lidocaine hydrochloride with epinephrine (Bimeda-MTC, Animal Health Inc., Cambridge, ON, Canada) given at the sacro-coccygeal or first intercoccygeal junction (Palomino et al., 2014a). A 16 French silicone embryo collection catheter (Bioniche Animal Health Canada Inc., Belville, Ontario, Canada) was placed in the uterine horn, and the cuff was inflated to fix the catheter in place. A catheter-tip syringe containing 20 – 30 mL of flush medium (Complete Flush, Bioniche Animal Health Canada Inc., Belville, Ontario, Canada) was connected to the collection catheter and used to flush the uterine horns. Each horn was flushed with 7 or to 8

syringe volumes and the contents of each flush were poured through a 75 micron filter (Emcon filter; Agtech, Manhattan, Kansas, USA). At the end of the flushing session, the filter was rinsed (Vigro Rinsing Solution, Bioniche Animal Health Canada Inc., Belville, Ontario, Canada) and poured into a 90 mm Petri dish for embryo indentification. Using a stereomicroscope (SMZ 1000, Nikon Instrument Inc., Melville, NY, USA) at a magnification of 8X, embryos were morphologically classified according to the guidelines of the International Embryo Transfer Society (Stringfellow and Givens, 2010). Freezable embryos were those classified according to the guidelines of the IETS (Stringfellow and Givens, 2010) as Grade 1 (≥80% of the embryonic mass intact, symmetrical and spherical, with blastomeres that are uniform in size, color, and density) and Grade 2 (≥50% of the embryonic mass should be intact, moderate irregularities in the overall shape of the embryonic mass or in size, color, and density of individual blastomeres).

6.3.6 Statistical analyses

The number of follicles 2 - 5 mm on Day 0 (wave emergence), follicles ≥9 mm on Day 5, CL on the day of embryo collection, and the number of embryos collected were compared among groups by analysis of variance and Tukey's post hoc tests in Experiment 1, and by t-tests in Experiment 2. The proportion of follicles that ovulated per bison (number of ovulations / number of follicles ≥9 mm on Day 5) was compared among groups by Chi square test. To evaluate the effect of season, data were combined among treatment groups in each season and compared (anovulatory vs ovulatory season) using t-tests. The number of follicles 2 - 5 mm on Day 0 was correlated with the number of follicles ≥9 mm on Day 5 using Pearson's correlation (r). The same bison were used for both experiments, but bison were assigned randomly to treatment

groups in each season. Data are presented as mean ± SEM, unless otherwise indicated. An event with a probability of ≤0.05 of happening by chance alone was considered statistically significant.

6.4. Results

6.4.1. Experiment 1 (anovulatory season, May-June)

There were no differences among groups in the number of follicles 2 - 5 mm on Day 0 or the number of follicles ≥9 mm on the day of hCG treatment (Day 5). The mean diameter of follicles ≥9 mm on Day 5 was smallest (P<0.01) in the progesterone group, and the proportion of follicles that ovulated per bison was lowest (P <0.05) in the group treated with progesterone + eCG (Table 6.1). Though not statistically different, the values for all remaining end points were numerically lower in the same group (progesterone + eCG; Table 6.1).

There were no ovulation failures in any group; i.e., all bison had ≥3 CL on the day of embryo collection. Transferable embryos were produced in 3 of 8 bison in the progesterone group, 7 of 9 bison in the eCG group, and 4 of 9 bison in the progesterone + eCG group. The distribution of ovulations is shown in Figure 2. A greater (P<0.05) proportion of ovulations were detected at 24 h vs. 48 h after hCG treatment in the progesterone and progesterone + eCG groups, whereas a greater (P<0.05) proportion were detected at 48 hr in the eCG group.

6.4.2. Experiment 2 (ovulatory season, September-October)

No differences were detected between groups for any end point, except the mean diameter of follicles ≥9 mm on Day 5 which was larger in the eCG group (Table 6.2). Ovulation was observed in all bison. One bison in the eCG group and two in the no-eCG group had ≤2 CL on the day of embryo collection. Transferable embryos were produced in 11/12 bison in the eCG

group and 10/12 in the no-eCG group. The proportion of ovulations detected at 24 and 48 h after hCG treatment did not differ within or between groups (Fig. 6.2).

6.4.3. Combined (anovulatory season vs. ovulatory season)

The number of follicles 2 - 5 mm on Day 0, number of follicles ≥9 mm on Day 5, and the number of CL at the time of embryo collection were greater ($P < 0.05$) in bison superovulated during the anovulatory season than during the ovulatory season (Table 6.3). The number of ova/embryos and embryos (all grades) were not different between seasons ($P = 0.61$ and $P = 0.80$, respectively); however, the number of freezable embryos (Grades 1 and 2) tended to be higher during the ovulatory season ($P = 0.06$). The number of follicles 2 - 5 mm on Day 0 positively correlated with the number of follicles ≥9 mm on Day 5 during both seasons (anovulatory season: $r = 0.69$, $P < 0.05$; ovulatory season: $r = 0.82$, $P < 0.05$).

Ovulation was observed in all bison during both seasons; 0/26 (0%) bison in the anovulatory season and 3/24 (12.5%) in the ovulatory season had ≤2 CL at the time of embryo collection. The proportion of ovulations detected at 24 and 48 h after hCG treatment did not differ within or between seasons (Fig. 6.2).

Table 6.1. Ovarian response and ova/embryo data (mean ± SEM) to superstimulatory treatment with or without the addition of eCG and exogenous progesterone in wood bison during the anovulatory season (Experiment 1).

Endpoints	Progesterone	eCG	Progesterone + eCG	P-value
Number of wood bison	8	9	9	
Number of follicles 2 - 5 mm on Day 0	16.6 ± 2.2	17.0 ± 4.5	18.6 ± 1.0	0.58
Number of follicles ≥9 mm on Day 5	14.8 ± 2.4	14.3 ± 4.6	16.0 ± 1.4	0.65
Mean size of follicles ≥9 mm on Day 5	10.7 ± 0.2 [a]	11.5 ± 0.2 [b]	11.2 ± 0.2 [b]	0.01
Proportion of follicles that ovulated per bison*	0.80 ± 0.07[a]	0.70 ± 0.05[a]	0.49 ± 0.08[b]	0.01
Number of CL	10.6 ± 1.7	9.7 ± 2.8	7.2 ± 1.0	0.23
Number of ova/embryos	5.4 ± 1.9	4.9 ± 2.3	2.9 ± 1.0	0.39
Number of embryos (all grades)	4.6 ± 1.6	3.8 ± 2.0	2.4 ± 0.7	0.41
Number of freezable embryos (Grades 1 and 2)	1.6 ± 0.9	1.0 ± 0.5	1.2 ± 0.3	0.48

* Number of ovulations / number of follicles ≥9 mm on Day 5
[a,b] Within rows, values with different superscripts are different (P<0.05)

Table 6.2. Ovarian response and ova/embryo data (mean ±SEM) to superstimulatory treatment with or without the addition of eCG in wood bison during the ovulatory season (Experiment 2).

Endpoints*	No eCG	eCG	P-value
Number of wood bison	12	12	
Number of follicles 2 - 5 mm on Day 0	12.3± 1.5	10.2 ± 1.3	0.48
Number of follicles ≥9 mm on Day 5	9.8 ± 1.5	9.1 ± 1.3	0.85
Mean size (mm) of follicles ≥9 mm on Day 5	11.1 ± 0.2	11.8± 0.3	0.05
Proportion of follicles that ovulated per bison*	0.82 ± 0.06	0.74 ± 0.08	0.38
Number of CL at the time of embryo collection	6.9 ± 1.1	5.4 ± 0.9	0.56
Number of ova/ embryos	4.2 ± 0.9	3.3 ± 0.5	0.49
Number of embryos (all grades)	3.7 ± 0.7	2.9 ± 0.4	0.47
Number of freezable embryos (Grades 1 and 2)	2.6 ± 0.7	2.0 ± 0.4	0.51

* Number of ovulations / number of follicles ≥9 mm on Day 5

Table 6.3. Effect of season on superovulatory response of superstimulated wood bison (mean ±SEM) during the anovulatory (Experiment 1) and anovulatory season (Experiment 2).

Endpoints	Anovulatory season	Ovulatory season	P-value
Number of wood bison	26	24	
Number of follicles 2 - 5 mm on Day 0	17.4± 1.7	11.3 ± 1.0	0.01
Number of follicles ≥9 mm on Day 5	15.0 ± 1.8	9.3 ± 1.0	0.01
Mean size of follicles ≥9 mm on Day 5	11.1 ± 0.1	11.4 ± 0.2	0.15
Proportion of follicles that ovulated per bison*	0.66 ± 0.08	0.78 ± 0.07	0.18
Number of CL on Day 13	9.1 ± 1.2	6.0 ± 0.7	0.01
Number of ova/ embryos	4.3 ± 1.0	3.6 ± 0.5	0.61
Number of embryos (all grades)	3.6 ± 0.8	3.2 ± 0.4	0.80
Number of freezable embryos (Grades 1 and 2)	1.3 ± 0.4	2.3 ± 0.4	0.06

* Number of ovulations / number of follicles ≥9 mm on Day 5

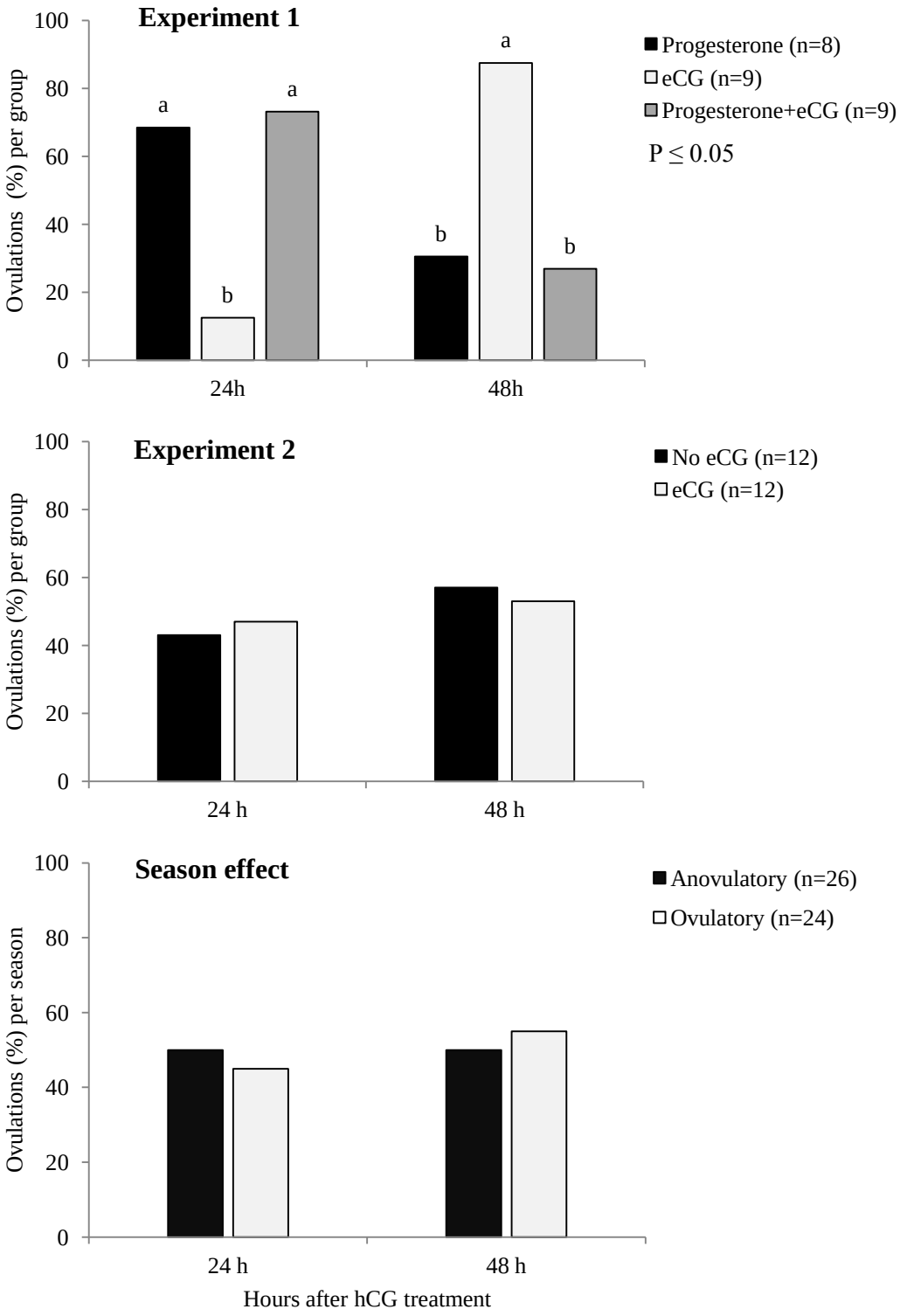

Fig. 6.2. Ovulations (%) detected in superstimulated wood bison at 24 h or 48 h after hCG treatment. Experiment 1 (top panel) was conducted during the anovulatory season and Experiment 2 (middle panel) during the ovulatory season. Data were combined among groups in each season to determine a season effect (bottom panel). A single dose of hCG (3000 IU) was given in all bison to induce ovulation. Values with different letters (a & b) are different (P≤0.05).

6.5. Discussion

The superovulatory response and embryo collection rates in the present study were higher than in any previous report in bison. In the present study, 26 superovulated wood bison produced 32 freezable embryos during the anovulatory season (1.3 per bison), and 24 bison produced 51 freezable embryos during the ovulatory season (2.3 per bison). The effect, however, was not attributable to either the inclusion of eCG or progesterone in the superovulatory protocol. Improvements in our knowledge of wood bison behavior and technical skills may have been contributed to the increase in embryo collection in the present work; an effect that has been reported in cattle (Robertson, 2015).

During both the anovulatory and ovulatory seasons, the number of CL as well as the number of embryos collected was numerically (though not significantly) lower in protocols that included eCG vs. no eCG. The rationale for the use of low doses eCG in more recent superstimulation studies in cattle is based on the idea that provision of LH activity will promote maturation and ovulatory capacity among the multiple superstimulated follicles, thereby minimizing persistent anovulatory follicles at the end of treatment (Mattos et al., 2011). A beneficial effect of replacing the last two doses of FSH in a superovulatory treatment with two doses of 200 IU of eCG each was reported in Nelore cows (Barros et al., 2007). Similarly, a greater number of ovulatory follicles was found in Sindhi cows after using two doses of 150 IU of eCG each (Mattos et al., 2011), and a greater number of transferable embryos were collected from *Bos indicus* cows with the addition of a single dose of 400 IU eCG (Reano et al., 2009) or in two doses (200 IU each; Cifuentes et al., 2009). Based on the results of the present study, however, bison are not responsive to supplemental eCG with respect to the superovulatory response or embryo production. Reasons for the difference between cattle and bison in this regard are unknown, but

our findings in bison are consistent with those of an early study in bison in which treatment with eCG for superstimulation resulted in only 2 CL per bison (Robison et al., 1998).

Paradoxically, mean follicle size at the end of superstimulatory treatment was greater in bison treated with eCG, but the proportion of follicles that ovulated was higher in bison that were not treated with eCG. Similarly in cattle, superstimulation with eCG (doses of 1500 to 2500 IU) induced larger preovulatory follicles compared to FSH treatment, but the ovulation rate was higher in FSH-treated cows (Monniaux et al., 1983; Goulding et al., 1996). The long half-life of eCG was associated with continued ovarian stimulation and follicle growth, consequently, a greater number of unovulated follicles (Mapletoft el a., 2002). Perhaps the dose of eCG used in the present study (400 IU) was sufficiently high to induce continued stimulation and follicle growth rather than maturation, resulting in increased ovulation failure.

The inclusion of progesterone during superstimulatory treatment tended to reduce the ovulatory response and the number of embryos collected. Test of the effect of progesterone in the present study was based on recent studies in cattle in which exogenous progesterone during the superovulatory protocol was associated with a greater number of transferable embryos in *Bos indicus* (Nasser et al., 2011) and *Bos taurus* (Rivera et al., 2011). The use of exogenous progesterone during superstimulation in cattle is apparently based on the concept that progesterone concentrations of <1 ng/ml during follicular growth may result in increased pulsatile release of LH, which may cause premature follicle and oocyte maturation and reduced embryo quality (Inskeep 2004). However, no such positive effect was observed in the present study or in a previous study in wood bison (Palomino Chapter 5). In the latter, number of embryos and freezable embryos did not differ in wood bison treated with or without exogenous progesterone. However, due to the low number of freezable embryos (0.6 to 0.7 per bison), a

follow-up study was needed to investigate the effect of progesterone with more embryos collected. The relative lack of progesterone and consequent increase in LH pulse-frequency may be pertinent in cattle, but it is noteworthy that the endocrine status of the bison during the anovulatory season is not analogous to that of post-partum or post-synchronized cattle. Although no reports were found on the seasonal differences in circulating gonadotropin concentrations in bison, we expect that LH is low during seasonal anestrus, as in other seasonal species (Robinson et al., 1985). In the ewe, circulating LH concentrations reach 8 ng/mL during the ovulatory season, but remain below 0.5 ng/mL in the anovulatory season (Legan et al., 1977; Robinson et al., 1985). Therefore, if LH was already low in bison in the anovulatory season, then exogenous progesterone may have suppressed it further. Consequently, follicle and oocyte maturation may have been affected due to the lack of LH to induce maturation at the end of the superstimulatory treatment.

The results of the present study also document distinct seasonal effects on ovarian response and embryo production in wood bison. The number of small follicles at the start of FSH treatment and the number of pre-ovulatory follicles at the end of treatment were greater in wood bison superstimulated during the anovulatory season than the ovulatory season. This result was unexpected, and the reason for the effect is unknown. In ewes, the peaks of FSH were more frequent during anestrous than the ovulatory season (Barret et al., 2004). The increased FSH availability may allow a greater follicle recruitment in anestrous ewes, which, indeed, occurred when the ewe was given 500 IU of eCG during the anestrous season vs. the ovulatory season (Barret et al., 2004). We may speculate that peaks of FSH were also greater in wood bison during the anovulatory season which allowed recruitment of more follicles during the emergence of the wave. Likewise, a strong correlation between the number small follicles at wave emergence and

the superovulatory response was detected in both seasons in the present study, and is consistent with studies in cattle where the number of follicles at wave emergence was predictive of the superovulatory response (Singh et al., 2004). A greater superstimulatory response in bison during the anovulatory season, therefore, is consistent with a greater number of small follicles at the beginning of FSH treatment in bison in the present study. Conversely, there was a tendency for the production of a greater number of freezable embryos during the ovulatory season in wood bison - an effect that has been described previously in ewes (Mitchell et al., 2002). Achievement of the oocyte competence at the time of induction of ovulation may explain these results. The pulsatile secretion of LH, along with estradiol support, are important for cytoplasmic maturation of the oocyte (Osborn and Moor, 1983; Oussaid et al., 1999). If circulating concentrations of LH and estradiol in wood bison are low during the anovulatory season, oocytes may have failed to achieve competence before ovulation. Therefore, while hCG treatment may have triggered ovulation, it may not have provided sufficient stimulus to induce cytoplasmic maturation in wood bison oocytes prior to ovulation.

In conclusion, the ovarian response and embryo production were not improved by the addition of eCG treatment near the end of the superstimulatory treatment protocol in either the anovulatory or ovulatory seasons. Additionally, exogenous progesterone did not improve the production rate of transferable embryos during the anovulatory season. The tendency for greater embryo production during the ovulatory season provides impetus for a follow-up study. Overall, the superovulatory response and embryo production in the present study were higher than any previous report in bison, but results were not attributable to the addition of either eCG or progesterone to the superstimulatory protocol.

6.6. Acknowledgements

The authors thank Dr. Elsie-Dawn Parsons for assistance with data collection, Bioniche Animal Health Canada Inc. for providing Folltropin-V and supplies for the embryo collection, Merck Animal Health for providing Chorulon, and Minitube of America for providing Triladyl for diluting the semen. We thank Elk Island National Park for donating the bison that now comprise the research herd at the Native Hoofstock Centre, University of Saskatchewan.

CHAPTER 7.

EFFECT OF EXTENDING FSH TREATMENT ON SUPEROVULATION AND EMBRYO PRODUCTION IN WOOD BISON (*BISON BISON ATHABASCAE*)

Relationship of this study to the dissertation:

In Chapter 6, we found no improvement in ovarian response with a treatment of a low dose of eCG in the superstimulion protocol and no benefit for the inclusion of an exogenous source of progesterone in the anovulatory season in wood bison cows. More importantly, in a comparison between seasons there is evidence of more high quality embryos during the ovulatory season. Therefore, in the present Chapter (Chapter 7), we investigated the effect of a modification (lengthening the period of FSH treatment protocol from 4 to 7 days) to induce a greater number of ovulations and, consequently, a greater number of embryos. It was hypothesized that the lengthened treatment would capture more small antral follicles and stimulate their growth to a stage of maturity that would enable them to respond to LH and ovulate. Results of the present chapter, along with the previous chapters, were expected to allowed us to understand whether superovulation would be a reliable technique that could be applied in wood bison to produce high quality (i.e., freezable) embryos.

7.1. Abstract

The effect of extending the FSH treatment protocol on superovulatory response and embryo production in wood bison was investigated during the anovulatory season (Experiment 1) and ovulatory season (Experiment 2). In Experiment 1 (anovulatory season), follicle wave emergence was induced by follicular ablation and bison were assigned randomly to two groups (n = 14 bison/group) and given 200 mg FSH in 0.5% hyaluronan i.m. on Day 0 (day after ablation) and Day 2 (non-extended group), or 133 mg FSH in 0.5% hyaluronan i.m. on Days 0, 2, and 4 (extended group). A single dose of hCG was given on Day 5 and Day 6 in the non-extended and extended groups, respectively, and bison were inseminated 12 and 24 hours later. Ova/embryos were collected 8 days after hCG treatment. In Experiment 2 (ovulatory season), bison (n=24) were given a luteolytic dose of prostaglandin, and wave emergence was induced by follicular ablation 8 days later. Bison were assigned randomly to two groups (n=12/group) and treated with FSH (non-extended or extended) and hCG as described for Experiment 1. Bison in both groups were given a luteolytic dose of prostaglandin on Day 3, inseminated 12 and 24 hours after hCG, and ova/embryos were collected 8 days later. Data were compared by t-test and Chi-square test. In Experiment 1 (anovulatory season), no differences were detected between the non-extended vs. extended treatment groups in the number of follicles ≥9 mm at the time of hCG treatment (10.4 vs. 10.1, respectively), proportion of follicles ≥9mm that ovulated (0.55 vs. 0.64, respectively), number of CL (4.4 vs. 5.5), ova/embryos (2.5 vs. 2.8), embryos (1.6 vs. 1.9), or freezable (Grades 1 and 2) embryos (0.4 vs. 0.2). In Experiment 2 (ovulatory season), there was no difference between the non-extended vs. extended treatment groups in the number of follicles ≥9 mm at the time of hCG treatment (7.4 vs. 9.1, respectively), proportion of follicles ≥9 mm that ovulated (0.60 vs. 0.66, respectively), or the number of CL (4.3 vs. 5.8, respectively).

However, a greater number of ova/embryos (2.3 vs. 4.3; P≤0.05) and a greater number of freezable embryos (1.2 vs. 2.5; P ≤0.05) were collected from bison in the extended group. In addition, the number of freezable embryos was greater in the ovulatory season than the anovulatory season (1.8 ± 0.4 and 0.3 ± 0.2, P≤0.05). In conclusion, extending the FSH treatment in wood bison did not improve the superovulatory response during the anovulatory season, but it did result in a greater number of freezable embryos during the ovulatory season. Additionally, more freezable embryos were produced during the ovulatory season; for practical purposes the ovulatory season may be preferred for embryo production.

7.2. Introduction

The largest reserve of wood bison (Bison bison athabascae) in the world, Wood Buffalo National Park in northern Alberta Canada, is endemically infected with tuberculosis and brucellosis (Joly and Messier, 2004a; 2004b; 2005). These diseases represent a risk of infection to healthy bison in and around the park (McCormack, 1992; Mitchell and Gates, 2002), and strategies to date have had no apparent effect on population growth or disease prevalence (McFarlane et al., 2006). Strategies, such as salvage techniques utilizing assisted reproductive technologies (i.e., superovulation, embryo collection, cryopreservation and transfer, etc), have been offered to eradicate the disease in the park, but are deemed cost-prohibitive (Shury et al., 2015). Likewise, eradicating the diseases by extirpation of infected herds is not tenable because the present population is less than 6% of historic numbers, and further decline may lead to irrevocable loss of genetic diversity (Wilson et al., 2005; McFarlane et al., 2006; COSEWIC 2013). Therefore, the development of techniques that will enable the preservation of valuable genetics will be important for management strategies to produce and preserve disease-free wood

bison. One such approach is the use of a genetic resource bank, as described for other wild species (Wild, 1992; Holt et al., 1999).

Superovulation and embryo collection are technologies that have been used to produce and preserve embryos of endangered and threatened wild species (Wild, 1992; Loskutoff et al., 1995; Solti et al., 2000). Embryos may be washed to remove potential contaminants from the zona pellucida (Stringfellow and Givens, 2010), and ultimately be preserved in a germplasm biobank as insurance against the loss of genetic diversity (Wild, 1992). The first attempts at superovulation in bison using the bovine model met with relatively poor results, attributed in large part to the stress of handling (Dorn et al., 1990, Matsuda et al., 1996; Robison et al., 1998; Othen et al., 1999). To minimize treatment-associated stress, we tested a simplified superovulation protocol in wood bison and found that two doses of FSH, given 48 hr apart, induced a greater ovarian response than four doses, given every 24 hours (Toosi et al., 2013). Furthermore, the response to a single dose of FSH diluted in 1% hyaluronan did not differ from the two-dose regime of FSH diluted in saline. In a subsequent study, we found that two doses of FSH diluted in 0.5% hyaluronan followed by a single dose of hCG effectively induced a superovulatory response during both the anovulatory and ovulatory seasons in wood bison (Palomino Chapter 5). Results of the most recent study revealed that the addition of a low dose of eCG to the superovulatory protocol did not improve the ovarian response or embryo collection rate in bison (Palomino, Chapter 6). Although the number of high quality embryos was greater than in any previous report in bison, the superovulatory response (6 to 9 CL) and the number of transferable embryos (1 to 2) remain relatively low.

In recent studies in cattle, extending the period of FSH treatment to 7 days induced a greater number of ovulations compared to conventional treatment (4 days; Garcia Guerra et al., 2012;

Dias et al., 2013a). The effect was attributed to the rescue and growth of small antral follicles within the follicular wave to a stage of maturity that enabled them to respond to LH and ovulate (Guerra et al., 2015). Perhaps a similar situation exists in wood bison, and extending the period of FSH stimulation will increase the superovulatory response, as described in cattle.

The objectives of the present study were 1) to test the hypothesis that extending the period of FSH treatment will increase the superovulatory response and embryo production in wood bison during both the anovulatory and ovulatory seasons, and 2) to confirm the effect of season on ovarian response and embryo collection rate in wood bison.

7.3. Materials and methods

7.3.1. Facilities and bison

The bison were maintained at the Native Hoofstock Centre, University of Saskatchewan (52°08'N, 106°38'W) with free access to alfalfa/brome grass hay and fresh water. The bison were between 7-11 years of age and had an average body condition score of 3.5 (scale of 1 to 5; Vervaecke et al., 2005). The bison were handled according to protocols approved by the University of Saskatchewan's Animal Research Ethics Board, in accordance with guidelines of the Canadian Council on Animal Care.

7.3.2. Experiment 1 (anovulatory season, June)

Wood bison were assigned randomly to two groups (n = 14 bison/group) to determine if extending the period of FSH treatment increases the ovarian response during the anovulatory season (Figure 1). Follicular wave emergence was induced in bison by ultrasound-guided aspiration of follicles ≥ 5 mm in diameter (follicular ablation), as previously described in wood

bison (Palomino et al., 2014a). In the non-extended group, 200 mg of FSH was given i.m. on the day after follicular ablation (Day 0, expected day of wave emergence), and on Day 2, followed by 3000 IU of hCG im (Chorulon, Merck Animal Health, Kirkland, Quebec, Canada) on Day 5. In the extended group, 133 mg of FSH was given i.m. on Days 0, 2, and 4, followed by 3000 IU of hCG i.m. on Day 6. For both groups, the FSH (400 mg, Folltropin-V, Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada) was diluted in 10 ml of 0.5% hyaluronan (5 mg/mL, MAP 5, Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada). The bison were inseminated with cooled semen (5^{o}C) 12 and 24 hours after hCG treatment. Ova/embryos were collected non-surgically 8 days after hCG treatment.

The ovarian response was assessed by transrectal ultrasonography using a 7.5 MHz linear-array transducer (MyLabFive VET, Biosound Esaote, IN, USA), as previously described in wood bison (McCorkell et al., 2013a; Palomino et al., 2014a). The ovaries were examined on Day 0 to record the number of follicles at wave emergence, and for three consecutive days beginning on the day of hCG treatment to detect ovulation. Ovulation was defined as the disappearance of a follicle ≥9 mm from one examination to the next. The ovaries were examined again on the day of embryo collection to record the number of CL.

7.3.3. Experiment 2 (ovulatory season, September)

Wood bison (n=24) were given a luteolytic dose of prostaglandin i.m. (500 μg Cloprostenol, Estrumate, Merck Animal Health, Quebec, Canada), and wave emergence was induced by follicular ablation 8 days later. Bison were assigned randomly to two groups (n=12/group) and treated with FSH (non-extended or extended) and hCG as described for Experiment 1. Bison in both groups were given a luteolytic dose of prostaglandin on Day 3, inseminated 12 and 24 hours

after hCG, and embryos were collected 8 days later (Fig. 2). The ovarian response was assessed by transrectal ultrasonography, as described in Experiment 1.

7.3.4. Semen collection and artificial insemination

Semen was collected from wood bison bulls (n = 3) by electro-ejaculation, pooled and diluted in commercial egg-yolk-based extender (Triladyl, Minitube of America, Canada), as previously described (Palomino Chapter 5). Diluted semen was preserved at 5^{o}C for ≤72 hrs before use. After warming to 37^{o}C, sperm motility, progressive motility, and concentration were, on average, 80%, 70%, and 300 x 10^6 sperm/ml, respectively. For insemination, chilled semen was loaded into a 0.5 ml straw (i.e., 150 x 10^6 sperm) and deposited into the uterine body using a standard AI gun (Palomino Chapter 5).

7.3.5 Embryo collection

Epidural anesthesia was induced with 4 to 5 mL of 2% lidocaine hydrochloride (Bimeda-MTC, Animal Health Inc., Cambridge, ON, Canada) given at or just caudal to the sacro-coccygeal junction (Palomino et al., 2014a). Embryo collection was carried out as previously described (Palomino Chapter 5). Briefly, using a stainless-steel stylet, a 16 French silicone catheter (Bioniche Animal Health Canada Inc., Belville, Ontario, Canada) was placed in a uterine horn, and the balloon cuff was inflated with 5 to 8 ml of flushing medium to fix the catheter in place and prevent reflux during flushing. Flushing of the uterine horn was done using a catheter-tip syringe containing 20 to 30 mL of collection medium (Complete Flush, Bioniche Animal Health Canada Inc.). The medium was instilled in the uterine lumen and aspirated during transrectal manipulation and agitation to recover the maximum of the instillate. Each uterine

horn was flushed with 7 or 8 syringe volumes of medium. The aspirates were poured into a 75 micron filter (Emcon filter; Agtech, Manhattan, Kansas, USA). After 7 to 8 flushes per uterine horn, the filter was taken to the lab to search and classify the embryos according to guidelines of the International Embryo Transfer Society (Stringfellow and Givens, 2010).

7.3.6 Statistical analyses

The number of follicles 2 - 5 mm on Day 0 (wave emergence), follicles ≥9 mm on the day of hCG treatment, CL on the day of embryo collection, ova/embryos, and freezable embryos were compared between groups by t-tests. The proportion of follicles that ovulated (number of ovulations / number of follicles ≥9 mm on Day 5) was evaluated by generalized linear mixed model. Freezable embryos were those classified according to the guidelines of the IETS (Stringfellow and Givens, 2010) as Grade 1 (≥80% of the embryonic mass intact, symmetrical and spherical, with blastomeres that are uniform in size, color, and density) and Grade 2 (≥50% of the embryonic mass intact, moderate irregularities in the overall shape of the embryonic mass or in size, color, and density of individual blastomeres). To evaluate the effect of season, the number of follicles ≥9 mm on the day of hCG treatment, CL, and embryos were combined within season and compared (anovulatory season versus ovulatory season) using t-tests. The number of follicles 2 - 5 mm on Day 0 was correlated with the number of follicles ≥9 mm on the day of hCG treatment using Pearson's correlation (r). Statistical analyses were done using the Statistical Analysis System (SAS version 9.2, Cary, NC, USA). Data are presented as mean ± SEM, unless otherwise indicated. A probability of ≤ 0.05 was considered statistically significant.

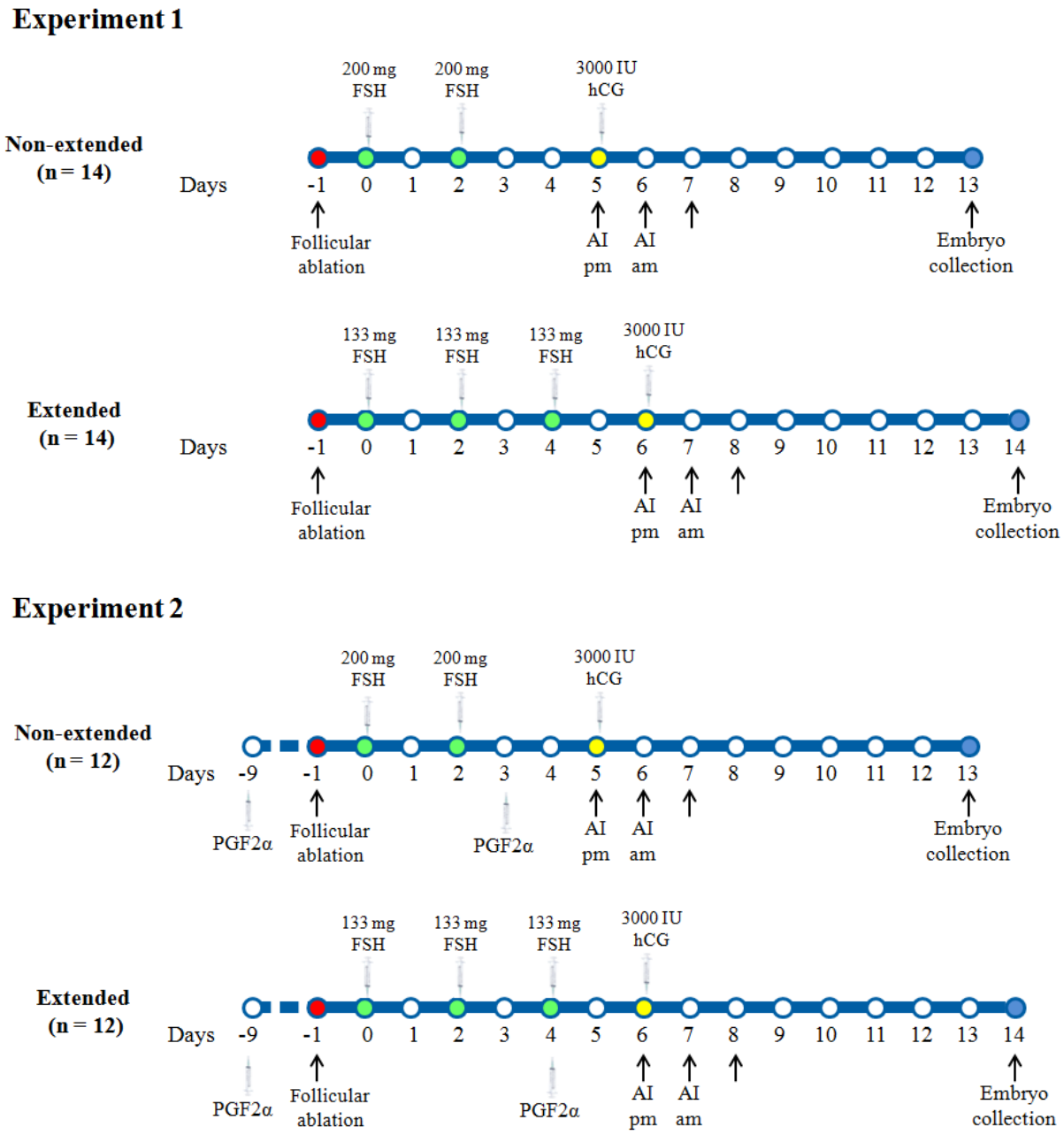

Figure 7.1. Superovulatory treatment and examination protocols for Experiment 1 (anovulatory season) and Experiment 2 (ovulatory season) in wood bison. Ovarian synchronization was done in bison by follicular ablation (FA). FSH diluted in 0.5% hyaluronan was given in bison on Days 0 and 2 (Non-extended groups), and Days 0, 2, and 4 (Extended groups). A single dose of hCG was given on Day 5 (Non-extended groups) and Day 6 (Extended group). Artificial insemination (AI) was done 12h and 24 h after hCG treatment. Embryo collection was performed 8 days after hCG treatment. Prostaglandin (PGF2α) was administrated during the ovulatory season 8 days before FA and on Day 3 (Non-extended group) and Day 4 (Extended group). Arrows: days of ovarian scanning.

7.4. Results

7.4.1. Experiment 1 (anovulatory season, June)

There was no difference between the non-extended and extended treatment groups for any end point (Table 7.1). One bison from each group failed to ovulate. Two bison from each group had ≤2 ovulations. The number of CL ranged from 1 to 10 in the non-extended group and 2 to 12 in the extended group. Freezable (Grades 1 and 2) embryos were produced from only 2 of 14 (14%) bison in the non-extended group and 2 of 14 (14%) bison in the extended group.

7.4.2. Experiment 2 (ovulatory season, September)

Follicle numbers before and after treatment, and the ovulatory response did not differ between the non-extended and extended treatment groups, but the mean diameter of follicles ≥9mm on the Day of hCG treatment was greater ($P \leq 0.05$) in bison treated with the extended FSH regime. Similarly, a greater number of ova/embryos and freezable embryos were collected from the extended group than the non-extended group ($P \leq 0.05$; Table 7.2). One bison from each group failed to ovulate. Two bison from the non-extended group and three from the extended group had ≤2 ovulations. The number of CL ranged from 1 to 8 in the non-extended group and 2 to 10 in the extended group. The proportion of bison that failed to produce a freezable embryo was 5 of 12 (42%) in the non-extended group and 3 of 12 (25%) in the extended group.

7.4.3. Season effect (Combined anovulatory season vs. ovulatory season)

Results are summarized in Table 7.3. The number of follicles 2 - 5 mm on Day 0 was greater ($P \leq 0.05$) and number of follicles ≥9 mm on the day of hCG treatment tended to be greater

(P=0.08) in bison during the anovulatory versus ovulatory season. There was a positive correlation between the number of follicles ≤5mm on Day 0 and number of follicles ≥9 mm on Day 5 during both the anovulatory (r = 0.52; P≤0.05) and ovulatory (r = 0.76; P≤0.05) seasons. The number of CL and number of ova/embryos collected did not differ between seasons, but the number of freezable embryos (Grades 1 and 2) was greater (P≤0.05) in the ovulatory vs. anovulatory season. The proportion of bison that failed to produce a transferable embryo was greater during the anovulatory season than during the ovulatory season (24/28 [86%] vs 8/24 [33%]; P≤0.05).

Table 7.1. Ovarian response and embryo collection (mean ±SEM) in wood bison treated with FSH on Day 0 (wave emergence) and Day 2 (non-extended protocol) or on Days 0, 2, and 4 (extended protocol) during the anovulatory season (Experiment 1).

Endpoints	Non-extended	Extended	P value
Number of bison	14	14	
Number of follicles 2 -5 mm (Day 0, wave emergence)	15.4 ± 1.5	16.1 ± 1.6	0.76
Number of follicles ≥9 mm (Day of hCG treatment)	10.4 ± 1.7	10.1 ± 1.1	0.89
Mean size of follicles ≥ 9mm (Day of hCG treatment)	10.8 ± 0.1	10.9 ± 0.2	0.60
Proportion of follicles that ovulated per bison*	0.55 ± 0.08	0.64 ± 0.08	0.42
Number of CL (Day of embryo collection)	4.4 ± 0.7	5.5 ± 0.9	0.35
Number of ova/embryos	2.5 ± 0.4	2.8 ± 0.7	0.68
Number of embryos (all grades)	1.6 ± 0.5	1.9 ± 0.7	0.70
Freezable embryos (grades 1 and 2)	0.4 ± 0.3	0.2 ± 0.2	0.57

* Number of ovulations/number of follicles ≥9 mm on Day 5

Table 7.2. Ovarian response and embryo collection (mean ±SEM) in wood bison treated with FSH on Day 0 (wave emergence) and Day 2 (non-extended protocol) or on Days 0, 2, and 4 (extended protocol) during the ovulatory season (Experiment 2).

Endpoints	Non-extended	Extended	P-value
Number of bison	12	12	
Number of follicles 2 - 5 mm (Day 0, wave emergence)	11.0 ± 1.2	12.8 ± 1.3	0.59
Number of follicles ≥9 mm (Day of hCG treatment)	7.4 ± 1.2	9.1 ± 1.4	0.37
Mean size of follicles ≥ 9mm (Day of hCG treatment)	10.8± 0.2	11.3 ± 0.2	0.05
Proportion of follicles that ovulated per bison*	0.58 ± 0.06	0.68 ± 0.07	0.14
Number of CL (Day of embryo collection)	4.3 ± 0.7	5.8 ± 1.0	0.26
Number of ova/embryos	2.3 ± 0.4	4.3 ± 0.8	0.04
Number of embryos (all grades)	2.0 ± 0.4	3.7 ± 0.8	0.07
Freezable embryos (grades 1 and 2)	1.2 ± 0.4	2.5 ± 0.6	0.03

* Number of ovulations/number of follicles ≥9 mm on Day 5

Table 7.3. Ovarian response and embryo collection (mean ±SEM) in wood bison superstimulated during the anovulatory season and ovulatory season.

Endpoints	Anovulatory season	Ovulatory season	P-value
Number of bison	28	24	
Number of follicles 2 - 5 mm (Day 0, wave emergence)	15.8 ± 1.1[a]	12.1 ± 0.8[b]	0.01
Number of follicles ≥9 mm (Day of hCG treatment)	10.3 ± 1.2	8.3 ± 0.9	0.08
Mean size of follicles ≥ 9mm (Day of hCG treatment)	10.9 ± 0.2	11.2 ± 0.2	0.11
Proportion of follicles that ovulated per bison*	0.59 ± 0.06	0.63 ± 0.06	0.65
Number of CL on day of embryo collection	4.9 ± 0.6	5.0 ± 0.6	0.88
Number of ova/embryos	2.7 ± 0.4	3.3 ± 0.5	0.51
Number of embryos (all grades)	1.8 ± 0.4	2.9 ± 0.5	0.06
Freezable embryos (grades 1 and 2)	0.3 ± 0.2[a]	1.8 ± 0.4[b]	0.01

*Number of ovulations/number of follicles ≥9 mm

7.5. Discussion

Extension of FSH treatment resulted in the production of a greater number of ova/embryos and freezable embryos (Grades 1 and 2) than the traditional shorter protocol only during the ovulatory season (Experiment 2). However, the number of preovulatory follicles at the time of hCG treatment and number of CL were not different between treatment groups, regardless of season. Therefore, our hypothesis that extending the period of FSH treatment will increase the superovulatory response and embryo production in wood bison throughout the year was only partially supported. In cattle, the number of ova/embryos and freezable embryos were not different in animals treated with an extended (FSH x 7 days) vs. a traditional short (FSH x 4 days) FSH treatment protocol (Garcia Guerra et al., 2012); however, numbers were numerically higher in the extended group. In the latter, the beneficial effect of the extended FSH regime was attributed to the ovulation of more follicles in the extended group (93%) than in the non extended group (66%). This suggests that despite being the same size, follicles in the extended group had reached a greater degree of maturity and were more able to ovulate. In our study, the effect may be attributed to a cumulative effect of numerically greater follicular response, ovulatory response (efficiency of ovulation and number of CL), and embryo collection rate.

In Experiment 2, there was a tendency for a higher proportion of preovulatory follicles that ovulated in the extended group than the non-extended group during the ovulatory season. Our findings agree with recent reports in cattle where extending the period of FSH treatment resulted in a greater number of ovulations than conventional treatment (Garcia Guerra et al., 2012; 2015). The ovulatory capacity changes during the growth of the dominant follicle in cattle (Bodensteiner et al., 1996); the number of LH-receptors per follicle were three times higher in 13 mm follicles compared to 8 to 10 mm follicles. Recently, we found that in wood bison, follicles

≥10 mm of diameter were more capable of ovulation in response to the gonadotropin signal (Palomino Chapter 4). This might be because larger follicles possess a greater number of LH receptors than the smaller follicles. In the present study, the mean size of perovulatory follicles was greater (P = 0.05) in the extended group than in the the non-extended group (11.3 ± 0.2 vs. 10.8 ± 0.2, respectively). Thus, having more LH-responsive follicles at the time of hCG treatment increases the likelihood of obtaining more ovulations.

A greater follicular diameter at the time of hCG treatment in the extended group may have resulted in greater maturation and more competent oocytes as has been found in cattle (Humblot et al., 2005). In a non-extended superovulatory scheme, small follicles (1 - 3 mm) that were rescued by the FSH have been shown to reach an ovulatory size (9 mm) at the time of LH or hCG treatment, but at least some lacked the capacity to ovulate (Garcia Guerra et al., 2012). If these follicles do not undergo maturation and ovulate, some elements of oocyte competence may fail (i.e., meiosis resumption, cytoplasmic maturation, etc.), which may result in the production of poor quality embryos (Sirard et al., 2006). Therefore, by extending the period of FSH treatment, we speculate that these rescued follicles will continue growing under the effect of exogenous FSH, giving them the opportunity to achieve maturation without adversely affecting oocyte quality.

In the ovulatory season, the mean size of follicles at the time of hCG treatment in the extended group was greater than in the non-extended group, which may explain the effect of FSH on the continuous growth of preovulatory follicles. Additionally, in cattle, a greater number of high quality oocytes (Grades 1 and 2) was obtained in superstimulated cows with an extended FSH treatment compared to a conventional shorter FSH regime (Dias et al., 2013a). We speculate that, a greater number of high quality oocytes (Grades 1 and 2) in the extended group

may have resulted in more freezable embryos. In the present study, differences in the number of freezable embryos (Grades 1 and 2) were found only during the ovulatory season. On the contrary, the numbers of freezable embryos were very low during the anovulatory season for reasons that remain unclear. In cattle, pre-exposure to progesterone was needed in cows before initiate the superstimulatory treatment (Nasser et al., 2001; Rivera et al., 2001). In bison, this issue has not been addressed.

There was a season effect on embryo quality; a greater number of high quality embryos was produced during the ovulatory season. This result is consistent with our previous findings (Palomino Chapter 6) where there was a tendency for more high quality embryos during the ovulatory season in wood bison. The detrimental effect of season on embryo quality has been reported in ewes (Mitchell et al., 2002b) and dromedary camels (Nowshari and Ali, 2005). There is no clear explanation for the seasonal effect, but it may be related to the low pulsatility of LH during the anestrous season in seasonal breeders (Robinson et al., 1985). The pulsatile secretion of LH may be involved in the cytoplasmic maturation of the oocyte during the late stages of the growing phase of the ovulatory follicle (Osborn and Moor, 1983; Oussaid et al., 1999). Since, the pulsatility of LH may be low during the anovulatory season in wood bison, follicles that ovulated after treatment with hCG may have produced oocytes that had not achieved complete cytoplasmic maturation. Further studies are required to test this hypothesis.

Interestingly, the number of follicles ≥9 mm at the time of hCG treatment tended to be greater during the anovulatory season than during the ovulatory season. In cattle, the number of follicles within a wave is related to the magnitude of the superovulatory response (Singh et al., 2004) and, the number of ova/embryos recovered (Ireland et al., 2007). Evidence for this may be found in seasonal differences in the number of follicles at the time of wave emergence in wood

bison. In the present study, number of follicles ≤ 5 mm on Day 0 (wave emergence) was greater (P≤0.05) during the anovulatory season than in the ovulatory season (15.8 versus 12.1, respectively). This could explain the tendency for a higher number of preovulatory follicles following treatment with FSH during the anovulatory season. However, this was not reflected in a higher number of ovulations or ova/embryos as might be predicted based on the studies in cattle (Singh et al., 2004).

A greater number of ova/embryos and freezable embryos were collected during the ovulatory season than in the anovulatory season. Similarly, in ewes, a greater number of Grade 1 and 2 embryos were obtained during the breeding season than in the early anovulatory season (Mitchell et al., 2002). The effect may be related to smaller dominant follicles (Sirard et al., 2006) in the anovulatory season (lower oocyte competence) than the ovulatory season. In this regard, despite more follicles at wave emergence and at end of superstimulation during the anovulatory season in this study, the numbers of ovulations and total number of ova/embryos collected was not different from the ovulatory season. Interestingly, despite a tendency for fewer ovulatory sized follicles during the ovulatory season, mean follicle size tended to be larger and this was reflected in a greater number of transferrable embryos. The slightly smaller follicle size during anovulatory season appeared to be associated with reduced oocyte competence (fewer fertilized embryos). Pulsatile LH secretion prior to the surge of LH secretion has been shown to be important in the induction of cytoplasmic maturation of the oocyte (Oussaid et al., 1999). We can speculate that in wood bison, the LH levels during the anovulatory season were very low and that this may have affected some elements of oocyte maturation and, consequently, production of competent oocytes and high quality embryos.

Overall, 28 superovulated wood bison produced 8 freezable embryos (Grade 1 and 2) during the anovulatory season whereas 24 superovulated females produced 40 freezable embryos during the ovulatory season. Our first attempts at collecting embryos from superovulated wood bison resulted in 5 transferable embryos collected from 20 females (0.3 embryos per bison, Toosi et al., 2013). Later, we obtained a 2-fold increase of freezable embryos (mean of 0.7 embryos) in wood bison Palomino Chapter 5). More recently, we were able to increase the number of freezable embryos to two embryos per superovulation attempt during the ovulatory season (Palomino Chapter 6). These results are very close to what we able to achieve in the present study where we obtained 1.7 freezable embryos per bison during the ovulatory season. Although the number of freezable wood bison embryos that we have been able to produce per superovulation attempt over the past few years has increased, it remains well below the number of transferable embryos produced in superstimulated cattle using simplified FSH treatments (6.1 embryos; Tribulo et al., 2012). In any event, it has been clearly shown in domestic cattle that high quality embryos will resume development following cryopreservation resulting in successful pregnancies. Cryopreservation has also been used to preserve gametes and embryos of endangered non-domestic species and to establish germplasm biobanks (Wildt, 1992; Solti et al., 2000). We are now in a very good position to develop freezing protocols for the preservation of high quality bison embryos (Grades 1 and 2) which will help in the reclamation of the threatened wood bison in WBNP.

During the anovulatory season (Experiment 1), there was no difference in the number of follicles ≥9 mm and ovulatory response in both groups (Non-extended and Extended). Interestingly, both superstimulatory treatments (non-extended and extended) were effective in inducing multiple follicle development (mean of 10 ovulatory follicles per bison). Recently,

Cervantes et al., (2013) showed that oocytes collected from superstimulated wood bison during the anovulatory season were competent to undergo *in vitro* and *in vivo* maturation. In addition, high quality embryos (Grade 1 and 2) were produced in vitro during the anovulatory season (Cervantes et al., 2015). The extended FSH treatment protocol used in this study may also be applied during the anovulatory season in wood bison for *in vitro* embryo production, as has been done in cattle (Dias et al., 2013b).

In conclusion, a greater number of freezable embryos (Grade 1 and 2) were collected following the use of the extended FSH treatment protocol during the ovulatory season. However, there was no effect on ovarian response or embryo collection of the extended FSH treatment during the anovulatory season. Obviously there is need to study this further in order to take advantage of the apparent benefits of this protocol. However, results of our study document that in vivo-derived embryos may be produced during both the anovulatory and ovulatory seasons, but based on current levels of application, the ovulatory season may be more productive.

7.6. Acknowledgements

The authors thank Brad McKell for assistance with data collection, Bioniche Animal Health Canada Inc. for providing Folltropin-V and supplies for the embryo collection, Merck Animal Health for providing Chorulon, and Minitube of America for providing Triladyl for diluting the bison semen. We thank Elk Island National Park for donating the bison that now comprise the research herd at the Native Hoofstock Centre, University of Saskatchewan.

CHAPTER 8.

EFFECTIVENESS OF WASHING PROCEDURES FOR REMOVING *BRUCELLA ABORTUS* FROM *IN VIVO*-DERIVED WOOD BISON EMBRYOS

Relationship of this study to the dissertation:

In Chapters 5, 6, and 7, protocols for superovulation and embryo collection in wood bison were developed and tested. The next step was to ensure that in vivo-derived embryos are free, or can be made free, of cattle diseases. Brucellosis, in particular, is a disease that has the potential to be transmitted through the reproductive system of cattle and bison, and this disease is of major concern in endangered herds of bison. Although it has been shown that early stage cattle embryos can carry this pathogen on its surface, there have been no studies in bison. To avoid the risk of transmission of Brucella organisms on bison embryos, we investigated the effectiveness of the 10-step washing procedures, recommended by the International Embryo Transfer Society, on in vivo-derived embryos exposed to the pathogen in vitro. Results of this Chapter (Chapter 8) allowed us to validate the washing procedures as an effective means of removing Brucella from in vitro-infected embryos. This study may be an important milestone for the entire bison project, because it provides a reliable tool that can be used in the field to disinfect embryos collected from Brucella-infected animals.

8.1. Abstract

The aim of the present study was to determine the effectiveness of washing procedures for removing *Brucella* bacteria from *in vivo*-derived wood bison embryos exposed *in vitro* to the pathogen. The study was done in 6 replicates (n = 4 bison per replicate). Embryos were collected from Wood bison after superovulation, and those with an intact zona pellucida were divided equally and placed into two 30 mm Petri dishes containing 2.7 mL of holding medium. *Brucella abortus biovar 1* (approximately $1x10^9$ CFU/mL in 0.3mL) was added to each dish and incubated for 2 h at 37°C in 8% CO_2. In each replicate, holding medium was cultured before exposure (negative control) and after incubation (positive control). After incubation, embryos were subjected to a 10-step washing procedure using wash medium either without antibiotics (PBS + 0.4% BSA) or with antibiotics (100 IU/mL penicillin and 100 mg/mL streptomycin). The wash medium was cultured at wash steps 1, 3, 6, and 9. After the 10^{th} wash, embryos were cultured individually on sheep blood agar and specific identification of *Brucella* organisms by PCR. *Brucella abortus* was not detected in negative control medium bit it was detected in the positive control medium. However, *Brucella* organisms were not detected in wash media after the third wash in either group (with or without antibiotics). Although *Brucella abortus* was detected by PCR in two embryos from the group washed ten times in medium without antibiotics (2/27), all embryos washed ten times in medium with antibiotics were culture negative (0/27). In summary, *Brucella abortus* was removed from 92% of *in vitro*-exposed bison embryos using the washing procedure without antibiotics, and from 100% of *in vitro*-exposed bison embryos using the washing medium with antibiotics. Results validate the embryo washing procedure for producing *Brucella*-free in vivo-derived wood bison embryos.

8.2. Introduction

The Canadian Wood bison (*Bison bison athabascae*) is a threatened species due to historic genetic bottlenecks and endemic diseases (McFarlane et al., 2006; Hedrick, 2009). The largest wood bison population, and most important with respect to genetically diversity, is in Wood Buffalo National Park (Wilson et al., 2005). However, the Park's population has an endemic infection rate of 30% to 60% for both tuberculosis and brucellosis (Mitchell and Gates, 2002; Joly and Messier, 2004b). Genetic diversity is critical for population survival (McFarlane *et al*., 2006), because a loss of diversity limits the ability of a population to adapt to environmental change and may lead to inbreeding depression (Frankham, 2005; McFarlane et al., 2006). Some measures to control these diseases (e.g. selective culling) may have played a role in the population decline (e.g., eliminating wood bison from the WBNP) and loss of wood bison genetic diversity in the Park and there is a need to develop options for future management of Wood bison (McFarlane et al., 2006; Shury et al., 2015). One option is the use of reproductive technologies to produce disease-free embryos and gametes for the purposes of establishing a germ plasm bio-bank (Wild, 1992; Holt et al., 1999) from which healthy calves may be produced to preserve the genetic material of the threatened Canadian wood bison.

A necessary step in establishing a germplasm bio-bank is the ability to produce embryos. Superovulation and embryo collection are technologies that have been used to produce embryos in wild animals (Comizzoli et al., 2000) and domestic livestock (Hansen, 2014). Recent studies in our laboratory demonstrated that superovulation and embryo transfer is a feasible technique (Palomino Chapter 5, 6, and 7) and that we can obtain live wood bison offspring by using these procedures (Toosi *et al*., 2013). Since we now know how to produce embryos in wood bison, the next logical study is to focus on producing disease-free embryos from infected wood bison.

Brucellosis is an endemic disease caused by bacteria of the genus *Brucella* (Corbel, 1997). Aside from bison, it affects economically important agricultural livestock such as cattle and small ruminants. Symptoms of the disease include abortion, retained placenta, reduced milk yield, orchitis, and infection of the male accessory sex glands (Neta *et al*., 2010). Bacteria may be shed in semen, milk, uterine discharge, and aborted fetuses, all of which may transmit the disease to healthy animals through direct contact or through ingestion of contaminated feed or water (Neta et al., 2010). Importantly, brucellosis is a zoonotic disease that can cause serious debilitation and even death in humans (Young, 1995). People at particular risk are dairy farmers, slaughterhouse employees, veterinarians, and laboratory technicians. Infected people display chronic intermittent fever (undulant fever), sweating, anorexia, fatigue, weight loss, and depression (Young, 1995). Because of the seriousness of this disease in animals and humans, it is considered a major reportable disease by the CFIA and the official status of "*Brucella*-free" has important implications for national and international trade. Canada has been considered *Brucella*-free in domestic cattle since 1985 (Environmental assessment panel, 1990). However, sporadic and isolated cases of the disease have occurred in some regions of Canada, suggesting that brucellosis remains in some wildlife reservoirs as reported in other countries (Olsen, 2010). In Canada, wood bison represent the main reservoir of brucellosis (Tessaro, 1986).

Although bovine fetuses can be infected with *Brucella abortus* via placental transport from maternal circulation, it is unknown if embryos in early stages can carry this pathogen (Stringfellow *et al*., 1984). In any event, to avoid the risk of transmission of *Brucella sp*, procedures for handling and cleaning potentially infected embryos may be used as recommended by the International Embryo Transfer Society (Stringfellow and Givens, 2010). Embryo washing is one of several procedures (e.g. enzymatic treatments, immunological methods) studied and

developed for removing infectious agents from embryos (Bielanski, 2007). Using medium containing antibiotics, the procedure involves serial transfer of embryos from one dish-well to another containing clean medium (dilution factor at least 1:100). Bacterial loads are expected to decrease to undetectable levels by the 10^{th} wash (Bielanski, 2007; Stringfellow and Givens, 2010). There is no information on the use of the washing technique to disinfect naturally or artificially infected bison embryos.

The present study was designed to determine the effectiveness of washing procedures with or without antibiotics for removing *Brucella abortus* from wood bison embryos previously exposed *in vitro* to the bacteria.

8.3. Materials and methods

8.3.1. Bison and facilities

The study was conducted at the Native Hoofstock Centre, University of Saskatchewan, Saskatoon during the months of September and October, using healthy wood bison cows (n = 24) between 6 to 10 years of age. The wood bison were originally caught from the wild at Elk Island National Park (53°36′52″N; 112°51′58″W), and moved to the Native Hoofstock Centre at 18 months of age. The bison were handled according to protocols approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

8.3.2. Superovulation and embryo collection

Ovarian synchronization and superovulation were induced in disease-free bison cows for each of six replicates (n=4 bison per replicate). To synchronize ovarian follicular and luteal

status prior to superstimulation, bison were given prostaglandin $F_{2\alpha}$ analogous (500 µg cloprostenol, Estrumate, Merck Animal Health, Quebec, Canada) intramuscularly at random stages of the estrous cycle and transvaginal ultrasound-guided follicular ablation was conducted 8 days later, as previously described (Palomino et al., 2014a). Ovarian superstimulation treatment was initiated the day after follicle ablation and consisted of a total dose of 400 mg of pFSH (Folltropin-V, diluted in 10 ml of 0.5% [5 mg/mL] hyaluronan, Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada) divided equally into 3 doses (Extended group) and given intramuscularly on Days 0, 2, and 4 (Day 0 = day after ablation) or into 2 doses (Non extended group) and given on Days 0 and 2. A single dose of 3000 IU of hCG (Chorulon, Merck Animal Health, Kirkland, Quebec, Canada) was given intramuscularly on Day 6 in the extended group and Day 5 in the non extended group. In both groups, bison were artificially inseminated 12 and 24 hours after hCG treatment. Embryos were collected 8 days after hCG treatment.

Embryos were collected from superstimulated bison (n=4 per replicate) on Day 14 using a non-surgical approach, as reported previously (Palomino Chapter 5). Each uterine horn was flushed (Complete Flush, Bioniche Animal Health Canada Inc., Belville, Ontario, Canada) and the aspirate was passed through a 75 micron filter (Emcon filter; Agtech, Manhattan, Kansas, USA). The contents were rinsed from the filter (Vigro Rinsing, Bioniche Animal Health Canada Inc., Belville, Ontario, Canada) into 90 mm Petri dishes. Flushing and rinsing media contained antibiotics. Embryos were located by stereomicroscopy at 10X magnification and classified at 100X magnification according to guidelines for cattle of the International Embryo Transfer Society (Stringfellow and Givens, 2010). Embryos were maintained and transported to the level 3 laboratory in holding medium without antibiotics (Dulbecco's phosphate-buffered saline + 2% fetal calf serum) at 37^{o}C. Embryos with an intact zona pellucida and free of adherent material

were pooled and placed in a cryo-vial with holding medium without antibiotics and transported to a Containment Level 3 laboratory at Intervac/VIDO, University of Saskatchewan for exposure to *Brucella* bacteria.

8.3.3. Preparation of Brucella strain

Embryo contamination and washing procedures were performed in a containment level 3 laboratory (Intervac/VIDO, University of Saskatchewan). The *Brucella abortus biovar 1* was obtained from the Canada Food Inspection Agency. The bacteria were streaked on 5% sheep agar (Fisher Scientific, #OXMP0105) plate and incubated at 37ºC in 8% CO_2 for at least 48 hours. Using a sterile swab, growth from the *Brucella* agar plate was transferred into a sterile 50 mL tube with 5 mL of holding medium. The absorbance was read at an optical density of 600 nm, and the *Brucella* sample was diluted with holding medium until reaching a concentration of $1x10^6$ to $1x10^8$ cfu/ml (= exposure medium).

8.3.4. Prewashing procedures and Brucella exposure

At the Level 3 lab (Intervac/VIDO), embryos were transferred to a 35 mm Petri dish with 200 uL holding medium. Embryos were washed 5 times in holding medium without antibiotics to remove any contaminants, microorganisms, or antibiotics (from the flushing medium) before exposure to *Brucella*. This washing procedure was done in a 6-well dish (Partnar Animal Health, Ilderton, Ontario, Canada) following the recommendations of the International Embryo Transfer Society (Stringfellow and Givens, 2010). Briefly, embryos (≤10 embryos/wash) were gently drawn from the Petri dish in 15 µL of medium using a 1-20 µL pipette and placed into the first well containing 1.5 mL of holding medium (Dilution 1:100). Then, 20 µl of medium was drawn

into the pipette tip from one edge and expelled in the opposite edge of the well to swirl the embryos and wash them. Then, washed embryos were drawn into the tip along with 10 µL of medium from the first well using a new sterile tip with 5 µL of medium from the second well,. Embryos were then transferred to the second well and washed as described for the first well. The process was continued through the five wells. Then, the integrity of the zona pellucida was evaluated under steromicroscope and only embryos with an intact zona pellucida were selected for the *Brucella* exposure procedures.

Prewashed embryos were divided equally into two groups consisting of wash medium with or without antibiotics. Prior to transferring embryos, a sample of the exposure medium (consisting of holding medium) was taken and cultured (*Brucella* negative control). Embryos were then transferred to two exposure dishes (35 mm Petri dish) containing 2.7 ml of holding medium each and taken to the Isolation room. There, dishes were placed into a class II biosafety cabinet where 0.3 ml of 1 x 10^9 CFU/ml of *Brucella abortus biovar 1* (the most common *Brucella* strain in the WBNP, Tessaro et al., 1990) suspended in embryo holding medium was added to each dish (final exposure concentrations are displayed in Table 8.2) and Petri dishes were then incubated at 37ºC in a 8% CO_2 incubator for 2 hours.

Post-incubation evaluation of embryo morphology and the integrity of the zona pellucida was obviated by the requirement of personal protective equipment in the Containment Level 3 laboratory, which included a full-face powered air-purifying respirator.

8.3.5. Embryo washing procedures

After incubation, embryos from each group were subjected to a 10-step washing procedure according to the IETS guidelines (Stringfellow and Givens, 2010). In the group without

antibiotics, the wash medium consisted of Dulbecco's phosphate-buffered saline + 0.4% bovine serum albumin whereas in the group with antibiotics, 100 IU/ml penicillin and 100 μg/ml streptomycin sulfate were added to the wash medium. Once embryos were transferred from the exposure medium to the first well, a sample of the remaining exposure medium was cultured (positive control). The washing procedure was done using the same technique as described above in the prewashing step. Wash medium from wash-steps 1, 3, 6, and 9 were cultured.

After the 10^{th} and final wash, each embryo was placed in a drop of 100 uL of fresh wash medium in a 90 mm Petri dish. Under a stereomicroscope, the zona pellucida of each embryo was physically ruptured using a glass pipette with a narrow tip (with a diameter slightly smaller than an embryo). Using a rubber bulb attached to the pipette, the embryo was aspirated in and expelled through the narrow tip several times until the zona pellucida was broken. Afterwards, the entire drop (100 ul) containing the broken embryo was cultured.

8.3.6. Brucella culture

Brucella growth was determined by culturing the samples in a commercial *Brucella* agar plate with 5% sheep blood (Fisher Scientific #OXMP0105). Using a sterile loop, the first third of the plate was streaked carrying the sample (i.e. holding medium, wash medium, embryos) along in a zigzag pattern. Starting in the previously streaked section, the other two thirds of the plate were streaked again as in a zigzag pattern. Plates were incubated at 37ºC in 8% CO_2 for 5 days and examined. *Brucella* positive colonies were those presented with non-hemolytic, non-pigmented small grey punctate colonies. To confirm presence of *Brucella* in colonies, DNA was extracted from the colonies and examined by standard PCR involving specific primers for *Brucella abortus biovar* 1.

8.3.9 Statistical analyses

The number of culture and PCR *Brucella* positive samples were compared between groups washed with antibiotics vs. without antibiotics by Fisher's exact test. A probability of less than 0.05 was considered statistically significant. The SAS version 9.2, Cary, NC, USA was used to perform the statistical analyses.

8.4. Results

A total of 65 ova/embryos were collected from 24 superovulated wood bison cows. Their distribution based on categories and treatment groups are summarized in Table 1. A total of 54 zona pellucida intact ova/embryos were used for *Brucella* exposure and washing procedures (n=27 per group).

Results of *Brucella* culture are shown in Table 2. No colony-forming units (CFU) grew in any of the negative control samples in the two treatment groups (wash medium with or without antibiotics). Conversely, too numerous to count CFU characteristic of *Brucella sp* were cultured after incubation of the positive control samples in both treatment groups. *Brucella abortus* was not detected in wash media cultured after the third wash in either group (with or without antibiotics). Two embryos from the group washed in medium without antibiotics (2/27) were positive for *Brucella* on culture and PCR, whereas all embryos washed in medium with antibiotics were culture and PCR negative (0/27). No statistical differences were found between treatment groups ($P = 0.24$).

Table 8.1. Ova/embryos collected from superovulated wood bison and their distribution based on categories and treatment groups.

	Total ova/embryos collected per category				ZP intact embryos used per treatment group (wash medium with or without antibiotics)			
Replicate	ES	M	B	UFO	Without antibiotics	n	With antibiotics	n
I		3	2		1M, 1B	2	1M, 1B	2
II	1	4	3	2	1ES, 2M, 1B	4	2M, 2B	4
III	2	2	5	1	1M, 3B	4	1ES, 1M, 2B	4
IV	5	4	1	2	1ES, 2M, 1B	4	2ES, 2M	4
V	7	4	1	2	4ES, 2M	6	3ES, 2M, 1B	6
VI	7	4	3	1	3ES, 2M, 2B	7	4ES, 2M, 1B	7
Total	22	21	15	8	9ES, 10M, 8B	27	10ES, 10M, 7B	27

Replicate = Day of embryo collection (n = 4 cows/replicate)
ES = Early stages (8 - 16 cells)
M = Morula
B = Blastocyst
UFO = Unfertilized ova
ZP = Zona pellucida

Table 8.2. *Brucella abortus* biovar 1 isolation from wash media and *in vitro* infected embryos collected from superovulated wood bison.

Replicate	Exposure dose of Brucella (CFU/mL)	Wash 1	Wash 3	Wash 6	Wash 9	Washed embryos	Number of culture-positive embryos
Wash medium without antibiotics							
I	5.8×10^5	P	N	N	N	2	0
II	7.5×10^6	P	N	N	N	4	0
III	9.4×10^5	P	N	N	N	4	0
IV	1.0×10^6	P	N	N	N	4	1
V	2.5×10^8	P	P	N	N	6	1
VI	1.9×10^8	P	P	N	N	7	0
Total						27	2
Wash medium with antibiotics*							
I	1.4×10^6	P	N	N	N	2	0
II	1.9×10^5	P	P	N	N	4	0
III	7.5×10^5	P	N	N	N	4	0
IV	5.6×10^5	P	N	N	N	4	0
V	1.4×10^8	P	P	N	N	6	0
VI	3.8×10^8	P	P	N	N	7	0
Total						27	0

Replicate = Day of embryo collection (n = 4 cows/replicate)
CFU = Colony-forming unit
P = Sample positive to *Brucella* bacteria after culturing and PCR evaluations.
N = Sample negative to *Brucella* bacteria after culturing and PCR evaluations.
*Antibiotics: 100 IU/ml penicillin and 100 μg/ml streptomycin sulfate

8.5. Discussion

Overall, 96% of embryos were disinfected from *Brucella* infection after washing the embryos with wash medium with (100%) or without (92%) antibiotics, respectively. These results support the hypothesis that the embryo washing procedure is an effective technique for removing *Brucella* bacteria from wood bison embryos exposed *in vitro* to the bacteria, especially when antibiotics are added to the wash medium.

The importance of our findings is that even though Canada is considered free of brucellosis in domestic animals, the bison in the WBNP are still infected with this disease (Mitchell and gates, 2002). Although brucellosis in cattle has not been detected in Canada since 1989 (Reviewed in Shury et al., 2015), there is evidence of transmission of *Brucella abortus biovar 1* (which was isolated in wood bison in WBNP) to healthy *Bos taurus* cattle (Forbes and Tessaro, 1996). Therefore, considerable resources go into regular testing and risk assessment for brucellosis in slaughterhouses and cattle auction markets in western Canada (Canadian Food Inspection Agency, 2015). Recently, a bovine cow from a farm near the Yellowstone National Park, Montana, USA, tested positive for brucellosis (The Western Producer, 2014). The source of infection was apparently elk or bison in the park. Therefore, the risk (the probability that an event will occur; Andersen et al., 2012) of spread of brucellosis from bison to cattle is very real. In Canada, the high prevalence of brucellosis in the free-roaming bison population of the WBNP and its proximity to bison and livestock farms, and free-ranging disease-free bison, represent a high risk of spreading the disease to healthy cattle and bison herds (Tessaro et al., 1990). In the present study, we have demonstrated that the 10 step washing procedure recommended by the IETS can eliminate *Brucella* bacteria from *in vitro*-infected wood bison embryos and,

presumeably in vivo-infected embryos and thus, it can be used to control disease transmission with embryos derived from animals in the WBNP.

Brucella bacteria infection of embryos via the uterus has been the subject of debate for many years (Barrios et al., 1988, Del Campo et al., 1987). The cow usually acquires *Brucella* through the nasal and oral mucosa, and from there the bacterium is taken up by the regional lymph system to local nodes (Poester et al., 2013). Later, a bacteremia occurs and Brucella infects target organs (i.e., uterus, mammary glands, etc.) and associated lymph nodes (Neta et al., 2010; Poester et al., 2013). In pregnant cattle artificially infected with *Brucella* Strain 2308 (Xavier et al., 2009) and *Brucella abortus biovar 1* (Forbes and Tessaro, 1996), the bacteria were detected in endometrial tissue and lymph nodes associated with the uterus (i.e., internal iliac nodes). Interestingly, in naturally infected non-pregnant cattle, the pathogen was also found in the uterine tissue and its associated lymph nodes (Gallien et al., 1998). The latter supports the hypothesis that *Brucella* can potentially infect embryos located in the uterus of infected animals and may be transmitted to healthy recipients by embryo transfer techniques (Stringfellow and Givens, 2000). However, in an earlier study, no healthy recipient was positive to *Brucella* after transfer of embryos collected from seropositive cows (Del Campo et al., 1987). Although the authors did not report the disease status of seropositive cattle (i.e., history of abortions or sickness), the authors of another study done in cattle (Barrios et al. 1988) found no culture-positive embryos collected from *Brucella* positive cattle with history of abortion. However, the embryos in the latter study were frozen before culturing which may have affected the viability of any potential *Brucella* after thawing, as reported previously in cattle (64% reduction, Stringfellow et al., 1986). Hence, whether *Brucella* may be in the uterus of a sero-positive

female one week after estrus and/or be transmitted by embryo transfer procedures remains unclear.

Washing procedures have been developed to reduce the risk of transmission of pathogens through embryo transfer technologies (Bielanski, 2007; Givens et al., 2007). To perform the washing technique, the zona pellucida (ZP) must not be damaged or broken and it must be free of adherent material that might trap an infectous agent (Stringfellow and Givens, 2010). An intact ZP is an effective barrier against most if not all pathogens in cattle and prevents infection of the embryo itself (Van Soom, 2010; Stringfellow and Givens, 2010). However, little is known about whether the embryo can carry *Brucella* attached to its ZP. The external surface of the ZP is irregular with the presence of numerous pores (Ridell et al., 1993), which could potentially trap some pathogens. Nevertheless, transmission of *Brucella* through the pores may be ruled out because *Brucella* is much larger (1.5 μm) than the largest pore (300 nm) seen in the ZP of the bovine embryo (Vanroose et al., 2000). Interestingly, *Brucella abortus* has not been shown to attach to the ZP of bovine embryos (Reviewed in Stringfellow and Wright, 1989). This is the case for most infectious agents that have been exposed to *in vivo*-derived bovine embryos. However, *in vitro*-derived embryos and embryos from other species e.g., sheep, are likely to harbor infectious agents on the ZP (Wolfe et al., 1988).

In the present study, all *in vivo*-derived wood bison embryos (n = 27) that were washed with washing medium containing antibiotics were negative to *Brucella* after culture. Penicillin (100 IU/mL) and streptomycin sulfate (100 mg/mL) were used in this study based on recommendations by the IETS (Stringfellow and Givens, 2010). Our result agrees with those reported in cattle (Stringfellow et al., 1984) and swine (Jacques et al., 2004), where the addition of antibiotics to the washing medium of embryos infected with *Brucella* organisms *in vitro*

resulted in 100% Brucella-free embryos. Our findings are also supported by earlier studies in cattle (Stringfellow et al., 1988) and bison (Robison et al., 1998) where recipients receiving embryos collected from *Brucella* seropositive cows and submitted to the 10-step washing procedures with antibiotics failed to seroconvert. Therefore, our results confirm that antibiotics must be added to the washing medium for rendering mammalian embryos pathogen-free (Bielanski, 2007, Stringfellow and Givens, 2000). Conversely, in our study, 2 out of 27 embryos (7%) were positive for *Brucella* bacteria following washing without antibiotics. Although it could be assumed that the pathogen had adhered to the ZP of these washed wood bison embryos, we cannot be certain as we could not confirm that ZP remained intact during the washing procedures. In a study in cattle, only embryos with broken ZP were positive to *Brucella* after *in vitro* infection and washing with medium containg antibiotics (Stringfellow et al., 1984; 1986). These authors suggested that the bacteria were able to penetrate through the ZP, avoiding the removal mechanism of the washing procedures. Thus, we may speculate that the ZP of our two positive embryos may have interfered with the effectiveness of the washing procedure. The other alternative is that the washing itself was not completely effective to remove the *Brucella*, but the concentration of the bacteria was decreased substantially. In the medium with antibiotics, the few organisms that remained after washing procedures may have been killed or inactivated by the antibiotics. The same may not have been occurred in the medium without antibiotics.

In all 6 replicates of the present study, *Brucella* was not isolated in the wash medium of either treatment group (with or without antibiotics) after the third wash. These findings are consistent with those reported in cattle (Stringfellow et al., 1984) and pigs (Jacques et al., 2000). Likely, the washing procedures acted as a method of serial dilution which reduced the numbers of bacteria (Ben-David and Davidson, 2014). Apparently, in our study, after the third wash,

Brucella was no longer present in wash medium or its viability on the blood agar plates was reduced to non-detectable levels, as previously reported (Oliver, 2005).

In conclusion, the washing procedures were effective in removing *Brucella* bacteria from *in vitro*-exposed embryos collected from superovulated healthy wood bison cows. The beneficial effect of the procedure is absolute when antibiotics are added to the washing medium. Consequently, we suggest that 100% of *Brucella* can be removed from exposed embryo. However, intactness of the zona pellucida must be evaluated not only prior to the washing procedures, but also during and after completion of the washing technique to ensure that organisms are not trapped in the ZP or with trophoblast cells. All in all, our results validate the embryo washing technique for producing *Brucella*-free wood bison embryos

8.6. Acknowledgements

The authors thank Shirley Hauta and Tracey Thue for their help in the Containment Level 3 laboratory at the International Vaccine Center. The authors also thank to Andrzej Bielanski, Jose Perez-Casal, Todd Shury, Brett Elkin, and Murray Woodbury for all their suggestions and input in the design of the study. We also thank Bioniche Animal Health Canada Inc. for providing Folltropin-V and supplies for the embryo collection, Merck Animal Health for providing Chorulon, and Minitube of America for providing Triladyl for diluting the semen.

CHAPTER 9. GENERAL DICUSSION

The wood bison (*Bison bison athabascae*) is a threatened species due to historic genetic bottlenecks and cattle diseases (McFarlane et al., 2006; Hedrick, 2009). The largest population of wood bison in the country is found in Wood Buffalo National Park (WBNP; Mitchell and Gates, 2002), and the park also contains the majority of wood bison genetic diversity (Wilson et al., 2005). Unfortunately, the bison population in the WBNP remains infected with tuberculosis and brucellosis (Mitchell and Gates, 2002). Therefore, there is a latent risk of spread to healthy bison herds in and around the park (Gates et al., 2001; Mitchell and Gates, 2002). To avoid the spread of disease, eradication of the infected herds has been suggested (Environmental assessment panel, 1990). This solution remains controversial and opposed by some (Shury et al., 2015). However, if eradication occurs without other considerations, the valuable wood bison genetic diversity of the WBNP may be lost. Genetic diversity is critical for population survival (McFarlane et al., 2006). The loss of genetic diversity could limit the ability of a population to adapt to environmental changes and may lead to inbreeding depression and even extinction (Frankham, 2005; McFarlane et al., 2006). Therefore, the overall goal of the wood bison research program is to develop a germplasm biobank using reproductive technologies to preserve the genetic material of the threatened Canadian wood bison population. Among these reproductive technologies, superovulation and embryo collection allow us to produce a greater number of embryos from live animals subjected to superstimulatory treatments.

In the present thesis, a series of studies has been conducted to develop a suitable superovulatory and embryo collection protocol for wood bison. Recent studies in the Adams's laboratory have allowed us to understand the normal reproductive pattern of female bison

(McCorkell et al., 2013a), and to develop techniques for ovarian synchronization (McCorkell et al., 2010; Palomino et al., 2014a), fixed-time artificial insemination (Adams et al., 2009), and ovarian follicular superstimulation in bison (Palomino et al., 2013; 2014b). Thus, we were in a privileged position to develop subsequent reproductive techniques, such as superovulation and embryo collection, in wood bison. We took into account that the bison is a wild species, and reducing the stress of handling played an important role in the success of the experiments. Therefore, simplified methods of ovarian superstimulation based on studies in cattle (Bo et al., Tribulo et al) and our previous studies (Palomino et al., 2013; Palomino et al., 2014b; Toosi et al., 2013) were used to develop the superovulatory protocols.

Four studies reported in the present thesis were designed to test the hypothesis that the development and improvement of simplified methods of superovulation will allow us to obtain a sufficient number of high quality (Grades 1 and 2) embryos throughout the year. These embryos may be used ultimately to establish a germplasm bio-bank. However, embryos produced in the present thesis were used in a fifth study to validate procedures of removing *Brucella* bacteria from wood bison embryos. Results of the experiments will be discussed in the present chapter (Chapter 9).

9.1 Inducing ovulation in wood bison during the anovulatory season

In bison, superovulation and embryo collection have been performed mainly during the ovulatory season (Dorn et al., 1990; Robison et al., 1998; Othen et al., 1999; Toosi et al., 2013). In other seasonal breeders, superovulation has been attempted during the anovulatory season (goat, Menchaca et al., 2010; wapiti, McCorkell et al., 2013b), but there are no reports of such attempts in bison. The goal of performing superovulation and embryo recovery in a season other

than the ovulatory season is to maximize the production of embryos throughout the year. Otherwise, embryos will be available in only one period of the year (the ovulatory season) and their numbers are likely to be limited. In wood bison, we learned that during the anovulatory season there is a continuous growth of follicles in a wave-like fashion (McCorkell et al., 2013a; Palomino et al., 2014a). However, ovulation of the dominant follicle does not occur (McCorkell et al., 2013a). Because a superovulatory protocol requires the occurrence of ovulation, we designed an experiment to determine whether ovulation of those anovulatory follicles could be induced in non-superstimulated wood bison and to determine the effect of ovulation-inducing agent (pLH vs. hCG) on ovulation rate during the anovulatory season (Chapter 4).

In total, 23 out of 32 (72%) bison cows ovulated during the anovulatory season. In these cows, treatment with hCG resulted in a greater ovulation rate than treatment with pLH (94% vs. 50%, respectively). Although both gonadotropins have the ability to bind the same receptor on the follicle, variants in their structure may be responsible for the different ovulatory response in wood bison. The variants confer a longer half-life plus an approximate 5-fold increase in potency to hCG compared to LH (Choi and Smitz, 2014). Therefore, the longer half-life may result in hCG having a longer occupancy on the LH receptors than pLH (Matzuk et al., 1990, Filicori et al., 2005). In cattle, ovulation rate was also greater in cows treated with hCG rather than GnRH (Dahlen et al., 2011), but ovulation rate did not differ between cows given hCG versus pLH (Gordon, 2011).

Human chorionic gonadotropin is composed of α and β subunits (Matzuk et al., 1990). hCG and LH share the same 92 amino-acids in the α subunit, but they differ in the number of amino-acids in their β subunit; i.e., 121 amino-acids for LH and 145 amino-acids for hCG (Naylor et al., 1990). These sequences of amino-acids are highly conserved among species. However, the

amino-acid complement of the β subunit is retained in hCG, while in LH, 24 amino-acids are lost to generate the final 121 amino-acid sequence (Naylor et al., 1990). Consequently, structural differences and post-translational modifications confer greater stability and longer half-life to the hCG. The half-life of hCG is approximately 30 hours (Rizkallah et al., 1969, Schmitt et al., 1996), whereas the half-life of LH is around 60 minutes (Robertson et al., 1991). In spite of all these differences, hCG and LH share the same LH receptor (LHR) on the follicle (Ascoli et al., 2002). However, recent studies have shown that LH and hCG may not elicit the same activity after binding to the LHR (Galet and Ascoli, 2005). The signaling pathways that follows the LHR binding is mainly controlled by cAMP (Ascoli et al., 2002). Interestingly, hCG was found to be more potent in affecting the cAMP response than LH (Casarini et al., 2012), which may explain the greater potency (40-fold) of hCG than LH in inducing the production of progesterone in humans. Whether hCG induced a higher ovulation rate in wood bison due to its more potent effect on cAMP remains to be demonstrated

In the present study, we found that more bison ovulated when their dominant follicle was ≥10 mm in diameter (88%) than when the follicles were 8 to 9 mm (56%). Apparently, full ovulatory capacity in this species may not be attained until the dominant follicle reaches a diameter of 10 mm. Since interaction of hCG or pLH at the LHR triggers the chain of events leading to ovulation, we may speculate that amount of LHR on follicle cells (i.e., granulose and theca cells) would be related to the occurrence of ovulation. In cattle, the number of LH-receptors per follicle was three times higher in 13 mm follicles compared to 8 to 10 mm follicles (Bodensteiner et al., 1996). Therefore, greater occurrence of ovulation was expected in the larger follicles. In wood bison, measuring the number of LHR within follicles may be needed to test the hypothesis that larger follicles (i.e., ≥10 mm in diameter) possess a greater number of LH

receptors, and are thereby more capable of ovulation in response to the gonadotropin signal. Similar hypothesis was proposed in cattle (Sartori et al., 2001).

In addition, there are no reports in wood bison for inducing ovulation during the anovulatory season. In other seasonal breeders, such as the ewe, females were induced to ovulate after GnRH treatment for the purposes of artificial insemination with modest results (Reyna et al., 2007). Likewise, in a study of red deer (*Cervus elaphus*), ovulation did not occur after the administration of GnRH during the anovulatory season (McLeod et al., 2001). The problem with GnRH is that it needs to act on receptors in the anterior pituitary gland to induce a preovulatory surge of LH to elicit ovulation (Karsh et al., 1997), which may be blocked during the anovulatory season through the negative feedback exerted by estradiol (Karsch et al., 1993) or other factors. Since we used pLH and hCG in wood bison, we expected to obtain ovulations because of their direct effects on the follicle without the need for the endogenous LH surge. Additionally, in our work, we demonstrated that ovarian follicles are not only responsive to the ovulatory effect of gonadotropins, but also that the ovulated follicle can become a corpus luteum which produces progesterone during the anovulatory season. This is very important for developing a superovulatory protocol during the anovulatory season because progesterone is important for the support of early stages of embryo development, and ovarian response can be measured by counting the number of corpora lutea (Monniaux et al., 1983).

In summary, hCG was more effective than pLH in inducing ovulation in wood bison during the anovulatory season. The size of the dominant follicle was associated with ovulatory response to either agent, and CL development and function was also induced during the anovulatory season. Whether developmental competence of oocytes resulting from ovulations during the

anovulatory season in wood bison is sufficient to result in high quality embryos requires further investigation.

9.2 Development and improvement of superovulatory protocols in wood bison

Superovulation involves prevention of follicle selection and stimulation of growth and ovulation of multiple follicles and production of multiple embryos with a high probability of obtaining pregnancies (Gonzalez et al., 2002). In cattle, one of the major problems related to the technique is the substantial variability of the ovarian response (Monniaux et al., 1983). Therefore, success of the superovulatory protocol depends on several factors: the timing of gonadotropin treatment relative to follicular wave emergence, gonadotropin preparations, ovarian superstimulation, induction and synchrony of ovulation, insemination (i.e., timing and sperm quality), and embryo collection procedures (Gonzalez et al., 2002) plus several intangible factors such as nutrition, health parameters and various stress factors. In cattle, superovulation and embryo transfer have been used to improve and propagate high quality genetics with considerable success (Seidel, 1981). However, superovulation and embryo transfer are technologies that can be used for reclamation of threatened and endangered species as well (Wildt, 1992; Holt et al., 1999; Loskutoff et al., 1995; Solti et al, 2000). In Chapters 5, 6, and 7, we investigated the feasibility of various protocols for superovulation and embryo production in wood bison. Our goal was to establish a suitable protocol for superovulation that can be used throughout the ovulatory and the anovulatory seasons to produce high quality (Grades 1 and 2) embryos.

In Chapter 5, the ovulation-inducing effects of pLH and hCG were compared in superovulatory protocols for wood bison. In the previous study (Chapter 4), we found that hCG

induced a higher ovulation rate of single dominant follicles in wood bison cows during the anovulatory season. Therefore, we designed two experiments (Experiment 5.1 during the anovulatory season and Experiment 5.2 during the ovulatory season) to determine if hCG also induces higher ovulation rate in superstimulated bison cows. We found that based on numbers of CL a single administration of hCG induced a greater ovulatory response than pLH in both the anovulatory (6.6 versus 2.8) and ovulatory (6.3versus 3.8) seasons. As described in Chapter 4, the greater effect of hCG to induce ovulation in wood bison may be related to the longer period of availability and occupancy on the follicular cell receptors as well as the longer half-life of hCG. Likewise, a longer period of availability may have allowed follicles that were immature at the time of treatment sufficient time and stimulus to mature and gain the capacity to ovulate (Sirard et al., 2006). Perhaps hCG has a greater affinity for LH receptors than pLH in wood bison as reported for hLH in humans (Casarini et al., 2007). In any event, hCG was more efficacious in inducing ovulation throughout the year, and consequently, was used for the subsequent studies reported in this thesis.

Early attempts of superovulation in bison resulted in the production of 3.6 (Dorn et al., 1990) and 2 (Robison et al., 1998) corpora lutea per bison cow. Reasons for these suboptimal results have been attributed to the stress for handling due to prolonged superstimulatory treatment protocols (Dorn, 1995). In a stressful event, corticotropin-releasing hormone (CRH) is secreted in the paraventricular nucleus of the hypothalamus (Habib et al., 2001). The CRH ultimately controls the secretion of corticoids in the adrenal glands, and together they exert an inhibitory effect on GnRH and LH secretion (Chrousos, 1997; Habib et al., 2001). Consequently, reproductive physiology is affected in the individual during stress. Since bison is a wild species and likely more susceptible to stress of handling than cattle, we speculated that the inhibitory

effect of the CRH and cortisol affected the ovarian response of those bison in the early studies. Therefore, in Chapter 5, simplified protocols involving fewer treatments for ovarian superstimulation that were applied previously in wood bison in our lab (Palomino et al., 2013; 2014b; Toosi et al., 2013). In Experiment 5.2, the number of corpora lutea was greater in bison given two FSH doses versus single FSH dose (7.1 ± 0.9 vs. 3.0 ± 0.8, respectively). The ovarian response to the two doses of FSH in the present study (7.1 CL/bison) was similar to that of our previous study (Toosi et al., 2013) in wood bison treated with FSH twice daily for 4 days (7.3 CL/bison) or treated subcutaneously with a two doses of FSH (8.9 CL/bison). However, our results with two doses of FSH were greater than observed in an early study in plains bison in which FSH was given twice daily (3.6 CL/bison; Dorn et al., 1990). The lower response in the latter study may be attributed to the stress of handling due to the multiple FSH treatments. All in all, the two dose FSH regime produced the highest ovarian response with less stress of handling in wood bison. Consequently, this dosing regimen has been used for follow up studies of superovulation in wood bison. However, 7.1 CL/bison still remains below the mean number of CL reported in cattle using simplified two dose FSH protocol (15 CL/cow; Tribulo et al., 2012). Therefore, superovulation in wood bison at this time requires further study.

Overall, there was no difference in the number of ova/embryos in Experiments 1 and 2. Freezable embryos (Grades 1 and 2) were very low during the anovulatory (1.3 ± 0.5 and 0.6 ± 0.2, respectively) and the ovulatory (1.6 ± 0.4 and 0.7 ± 0.2, respectively) seasons in this study. Reasons for the low embryo recovery rates were not determined. These results still remained suboptimal because the main goal was to produce high quality embryos (Grades 1 and 2) that can be cryopreserved for reclamation of the wood bison sub-species. In water buffalo, it was hypothesized that high levels of estradiol during the superstimulation may impair the embryo

development and transport in the oviduct of superovulated females (Baruselli et al., 2000). However, *in vivo* and *in vitro* studies have not supported this hypothesis (Baruselli et al., 2002). Recently, the administration of PGF2α during the periovulatory period increased the number of embryos recovered in water buffaloes (Reviewed in Baruselli et al., 2013). The authors suggested that PGF2α improves transportation of embryos through the oviduct. Whether PGF2α treatment in wood bison may improve embryo collection needs to be investigated.

In Chapter 6, the effect of eCG on superovulation and embryo collection was tested to improve the ovarian response in wood bison and embryo quality. The eCG is a glycoprotein produced by the endometrial cups between Days 38 and 130 of pregnancy in the mare (Allen, 1969; Allen et al., 1973; Murphy, 2012). It has both FSH- and LH-like effects and has a long circulating half-life of 3 to 5 days in cattle (Stewart et al., 1976; Murphy and Martinuk, 1991). As with other glycoproteins (e.g., equine LH), eCG is composed of dissimilar α and β subunits non covalently complexed (Murphy and Martinuk, 1991). The β subunit of the eCG (β-eCG) differs from the equine β-LH because the glycosylated chains in the former contains mainly sialic acid whereas the latter is composed of sialylated and sulfated forms (Matsui et al., 1991). Sialic acid attachments are more stable than sulfated bonds (Martinuk et al., 1991). Consequently, deglycosylation for removing eCG from blood circulation is delayed, and thus, its half-life increases (Martinuk et al., 1991).

In cattle, eCG has been used to induce ovarian superstimulation (Monniax et al., 1983). Although eCG has the advantage of requiring only a single dose (2,500 - 3,000 IU/dose), its long circulating half-life (3-5 days) seems to be a considerable problem for superstimulatory treatments due to the induction of persistent follicles or early multiple ovulation (Monniaux et al., 1983; Mapletoft et al., 2002). More recently, the replacement of the two last doses of FSH

with two low doses of eCG (200 IU/ dose) in a twice-daily FSH protocol resulted in a greater number of ovulatory follicles (Barros et al., 2008; Mattos et al., 2011). The rationale for the use of low doses eCG is based on the idea that provision of extra LH activity will help the multiple follicles mature and reach the ovulatory capacity (Mattos et al., 2011). A greater number of ovulatory follicles may result in more number of CL. However, results in Chapter 6 have shown no effect of a low dose of eCG on ovarian response during both the anovulatory and ovulatory season in wood bison. Reasons for the difference between cattle and bison in this regard are inconclusive. We may speculate that there is a difference between species because in previous superovulatory protocols, where the low dose of eCG induced a greater ovarian response, the experiments were conducted in *Bos indicus* cows (Barros et al., 2008; Mattos et al., 2011). This hypothesis was not supported by a large study done in *Bos taurus* cows were the low dose of eCG did not improve ovarian response (Davis et al., 2011).

The effect of eCG on embryo recovery rate and embryo quality was also evaluated in Chapter 6. The number of ova/embryo and freezable embryos (Grades 1 and 2) was not affected by eCG during the anovulatory or ovulatory seasons. These results disagree with previous reports in cattle, in which a greater number of transferable embryos was observed in cows treated with two injections of eCG (200 IU/dose 24 hours apart; Reano et al., 2009) at the end of a twice-daily FSH protocol. There is no clear explanation why different results were observed in our study compared to those reported in cattle. It might be due to the species-specific differences between wood bison and cattle, but this hypothesis needs further investigation. Interestingly, the administration of 500 IU of eCG 2 days before initiating FSH treatments increased embryo production in problem donor cows (Reviewed in Bo et al., 2008). This approach should be investigated to increase ova/embryo production in wood bison.

The effect of progesterone for increasing the number of high quality embryos in wood bison during the anovulatory season was also tested in Chapter 5 and 6. However, no effect of exogenous progesterone on embryo quality was found in these studies. Conversely, supplementation with exogenous progesterone resulted in a greater number of transferable embryos in cattle (Nasser et al., 2011; Rivera et al., 2011). The rationale is that progesterone may prevent premature follicle and oocyte maturation by preventing high release of pulsatile LH during the follicular growth (Inskeep, 2004). The difference may be because the endocrine status of wood bison during the anovulatory season is different than cycling cattle. In seasonally anestrous ewes, LH levels remain far below those found during the ovulatory season (0.5 vs. 8.0 ng/mL, respectively; Legan et al., 1977; Robinson et al., 1985). Similar LH levels may be present in the anestrous wood bison cows. Therefore, exogenous progesterone may not be needed at all. Possibly there was insufficient replication to evaluate the effect of progesterone on embryo quality, and thus may need further investigation.

Although ovarian response and embryo recovery rates were higher in the study reported in Chapter 6, embryo quality was still below that reported in cattle. Therefore, the study reported in Chapter 7 was designed to improve not only the ovarian response, but also the embryo quality in superovulated wood bison. In Chapter 7, the effect of extending of FSH treatment (from two doses at 48 hour intervals to three doses at 48 hour intervals) on superovulatory response was evaluated during the anovulatory and ovulatory seasons. The rationale of this study was that the lengthening effect of FSH will provide additional time for small follicles to grow and acquire the capacity to ovulate (Garcia Guerra et al., 2012; Garcia Guerra et al., 2015). Therefore, having a greater number of ovulatory follicles may result in a greater number of ovulations and consequently a greater number of ova and embryos. Additionally, by extending the period of

FSH treatment, we may provide oocytes from follicles that were in the smaller size categories at the start of treatment the opportunity to acquire competence without affecting the quality of the already matured follicles. If follicles ovulate non-competent oocytes, the production of high quality embryos is likely to decrease (Sirard et al., 2006).

We found that extending the FSH treatment protocol in wood bison did result in a greater ovarian response and a greater number of high quality embryos, but only during the ovulatory season. The greater number of preovulatory follicles found in the extended group may have resulted in greater number of competent oocytes (i.e., molecular competence) in wood bison. In *in vitro* studies in cattle, larger follicles produced more competent oocytes resulting in more transferable embryos (Humblot et al., 2005). We speculate that in wood bison treated with the extended FSH protocol, follicles continued growing under the influence of exogenous FSH, giving their oocytes the opportunity to achieve cytoplasmic and molecular competence. Therefore, a greater number of competent oocytes in the extended group may have resulted in more freezable embryos . Interestingly, the effect of the extended FSH protocol was found only during the ovulatory season; freezable (Grades 1 and 2) embryos were very low during the anovulatory season for reasons that remain unclear. According to our results, low superovulatory response and embryo recovery contributed to obtain low number of freezable embryos during the anovulatory season.

A seasonal effect on embryo quality was found in wood bison (Chapter 7). The number of freezable embryos (Grades 1 and 2) were greater during the ovulatory season than in the anovulatory season (1.8 ± 0.4 vs 0.3 ± 0.2, respectively). The seasonal effect on embryo quality has been reported previously in sheep (Mitchell et al., 2002b) and dromedary camels (Nowshari and Ali, 2005). The low pulsatile levels of LH during the anovulatory season (Robinson et al.,

1985), may affect cytoplasmic maturation of the oocyte during the late stages of the growing phase of ovulatory follicles (Osborn and Moor, 1983; Oussaid et al., 1999). Interestingly, the slightly smaller follicle size during anovulatory season was not associated with a reduced ovulatory competence (e.g., proportion of follicles that ovulate was the same), but was associated with reduced oocyte competence (fewer fertilized embryos). Therefore, oocytes that were produced during the anovulatory season in wood bison may not have achieved complete cytoplasmic maturation and; consequently, produced low quality oocytes which we were unable to determine because of low ova/embryo production. We speculate that the oviductal environment during the anovulatory season may also not be adequate for sustaining the quality of embryos during their conveyance to the uterus. This hypothesis needs to be tested.

In summary, superovulation can be accomplished in wood bison during both the anovulatory and ovulatory seasons. A summary of our results from years 2012 and 2014 is displayed in Table 9.1. Extending the FSH treatment protocol (from two to three doses of FSH at 48 hour intervals) may be used to obtain a greater ovarian response and embryo numbers. Since, more high quality embryos (Grade 1 and 2) are produced during the ovulatory season, efforts to produce *in vivo*-derived embryos during this season should be emphasized. Freezable embryos (Grades 1 and 2) may be preserved in a potential wood bison germplasm bio-bank using this approach.

Table 9.1 Ovarian response and ova/embryo data (mean ±SEM) from years 2012 - 2014 in wood bison (n) superstimulated with simplified FSH protocols during the anovulatory and ovulatory seasons.

Endpoints	Anovulatory season	Ovulatory season	P-value
Bison (n)	48	44	
Number of corpora lutea	7.2 ± 0.7	5.9 ± 0.5	0.15
Number of ova/embryos	3.7 ± 0.6	3.5 ± 0.4	0.78
Number of embryos (all grades)	2.9 ± 0.5	3.1 ± 0.3	0.69
Number of freezable embryos (Grades 1 and 2)	0.8 ± 0.2	2.0 ± 0.3	0.001
Number of embryos preserved in the lab	28	46	

9.3 Production of disease-free wood bison embryos

The overall goal of the bison project is to produce disease-free embryos from diseased wood bison of the WBNP that can contribute to the preservation of genetic diversity. To achieve this goal we have been working on development of efficient techniques for removing pathogens from wood bison embryos. One of the pathogens infecting bison herds in the WBNP is *Brucella abortus* biovar 1 (Tessaro et al., 1990; Olsen and Tatum, 2010). This pathogen is the cause of the brucellosis in wood bison of the WBNP. Brucellosis, along with bovine tuberculosis, has been considered an important factor for the population decline of wood bison since the 1960s (Joly and Messier, 2001; Joly and Messier, 2004a). Brucellosis may induce abortion in affected animals (Davis, 1990; Joly, 2001; Joly and Messier, 2005), increase the risk of predation (Joly and Messier, 2004a), or affect body condition in such a way to decrease survival during severe weather conditions (Environmental Assessment Panel, 1990; Reynolds *et al*., 2003). There is also a latent risk of spread of the disease to healthy bison herds, domestic livestock, and even humans in proximity to diseased bison (Joly and Messier, 2004b, Nishi et al., 2006; Tessaro et

al., 1990). For these reasons, eradication of infected herds in the WBNP has been suggested (Environmental Assessment Panel, 1990), with possible consequences of losing the valuable genetic diversity found among these wood bison (McFarlane et al., 2006). However, without intervention, brucellosis will likely persist indefinitely in the WBNP. Therefore, we designed the final study in this dissertation (Chapter 8) to develop a technique for the removal of *Brucella* bacteria from wood bison embryos. The rationale for this work is that there is a potential of transmission of the pathogen through the reproductive system of infected animals to any embryos that they might produce (Forbes and Tessaro, 1990; Gallien et al., 1998). Consequently, there is a risk that the embryo can carry the bacteria from an infected donor to a healthy recipient cow in an embryo transfer program (Bielanski, 2007; Givens et al., 2007).

The International Embryo Transfer Society recommends the 10-step washing procedures for handling and cleaning potentially infected in vivo-produced embryos in cattle (Stringfellow and Givens, 2010). The procedure involves serial transfer of embryos from one dish-well with wash medium containing antibiotics to another containing clean medium with a dilution factor of 1:100. Bacterial loads are expected to decrease to undetectable levels by the 10^{th} wash (Bielanski, 2007; Stringfellow and Givens, 2010). The procedure has been effective for removing *brucella* bacteria infected *in vitro* from bovine (Stringfellow et al., 1984), sheep (Riddle et al., 1989), and porcine (Jacques et al., 2004) embryos.

In Chapters 5, 6, and 7, we had improved and simplified protocols for superovulation and embryo production in wood bison. The overall conclusion was that freezable embryos (Grades 1 and 2) can be collected from superovulated wood bison, and that more high quality embryos can be produced during the ovulatory season. Therefore, the follow up step was to ensure that these embryos are *Brucella* free before cryopreservation in a germplasm bio-bank. With this in mind,

the objective of Chapter 8 was to determine the effectiveness of washing procedures with or without antibiotics for removing *Brucella abortus* from zona pellucida intact wood bison embryos previously exposed to the bacteria *in vitro*. To our knowledge, this is the first to report the use of the washing procedure for producing disease-free embryos in wood bison.

Results of the experiment showed that *Brucella* exposed *in vitro* to *in vivo*-derived wood bison embryos can be removed by washing procedures. However, 2 out of 27 wood bison embryos, were positive to *Brucella* bacteria after 10 washes with media that did not contain antibiotics. We assume that the pathogen attached to the zona pellucida (ZP) of the two embryos and could not be removed by the washing procedures. However, technical difficulties during the procedures in the level 3 laboratory made evaluation of ZP impossible during and after washing. If the ZP of these two embryos were broken or compromised, the bacteria could have become trapped within it and remained sequestered, preventing its removal during washing. In cattle, embryos with broken ZP remained positive for *Brucella* after washing with a medium containing antibiotics (Stringfellow et al., 1984). The authors suggested that the bacteria were able to penetrate through the ZP, thus avoiding removal by the washing procedures. We speculate that a similar effect may have occurred with our two positive embryos. Another hypothesis is that both procedures (with and without antibiotics) left a few organisms attached to the zona pellucida and only the medium with antibiotics rendered the embryos disease free. On the contrary, *Brucella* was removed in 100% of the embryos washed with washing medium containg antibiotics. This finding agrees with results reported in cattle (Stringfellow et al., 1984) and pigs (Jacques et al., 2004). Therefore, our results confirm that antibiotics must be added to the washing medium to render mammalian embryos specified pathogen-free (Bielanski, 2007, Stringfellow and Givens, 2000).

Therefore, the ten step washing procedures is another technology that can be applied in the field for helping in reclamation of threatened wood bison in the WBNP. The technique is easy to apply in the field, requires little time to perform, and is done routinely by practitioners processing cattle embryos. We can collect *in vivo*-derived embryos from *Brucella*-infected superovulated wood bison and then apply the ten-step washing procedures for removing the bacteria from embryos. Washed embryos can then be cryopreserved and stored in germplasm biobanks for further use in reclamation of wood bison.

CHAPTER 10. GENERAL CONCLUSIONS

Overall, results of the present dissertation support our hypothesis the development and improvement of simplified methods of superovulation throughout the year will allow us to obtain a greater number of freezable (Grade 1 and 2) embryos. These embryos, ultimately were used to validate the washing procedures for removing *Brucella* bacteria from wood bison embryos.

Based in all our results we can conclude that:

- Induction of ovulation was more effective in cows treated with a single dose of hCG than pLH. The hCG was effective in wood bison cows with or without superstimulatory treatment and regardless of season.
- Treatment with a two dose of FSH in 0.5% hyaluronan resulted in a greater ovarian superstimulatory response than treatment with a single FSH dose in hyaluronan in wood bison.
- Ovarian response was improved by lengthening the FSH treatment protocol in wood bison cows. However, the addition of a low dose of eCG as a replacement for the last two FSH treatments did not have a beneficial effect on superovulation or embryo production.
- Exogenous progesterone (PRID) did not improve embryo quality following superovulation during the anovulatory season in wood bison.
- There was a seasonal effect on embryo quality collected from superovulated wood bison. More freezable embryos (Grades 1 and 2) were obtained during the ovulatory season. We suggest that the production of *in vivo*-derived embryos should be emphasized mainly in this season.

- Washing procedures were effective in removing *Brucella* bacteria from *in vitro*-exposed zona-intact embryos collected from superovulated healthy wood bison cows. The beneficial effect of the procedure was absolute when antibiotics were added to the washing medium. Our results validate the embryo washing technique for producing *Brucella*-free wood bison embryos

CHAPTER 11. FUTURE STUDIES

Results and conclusions presented in this thesis have allowed us to better understand superovulation and embryo production as techniques that can be used for preserving genetic material from the threatened wood bison. However, some questions arose during the preparation of this dissertation. Therefore, future studies must be done to:

- Determine the effects of ovulation induction agent and follicle maturity on the developmental competence of oocytes fertilized *in vivo* or *in vitro* during the anovulatory season.
- Investigate the apparent lack of an effect of progesterone on embryo quality in wood bison.
- Investigate the effect of eCG on ovarian response and embryo quality in bison treated with the lengthened protocol of FSH or use the low dose of eCG at the beginning of the superstimulatory protocol.
- Evaluate the survival rate and fertility potential of frozen/thawed wood bison embryos collected from superovulated cows during the ovulatory season.
- Evaluate the pregnancy and birth rate of fresh and frozen/thawed embryos transferred to plains bison recipients.
- Investigate the properties of *Brucella* and its potential to attach to the zona pellucida of wood bison embryos.
- Determine seroconversion of recipients receiving wood bison embryos exposed *in vitro* and *in vivo* to *Brucella* abortus and washed with medium with antibiotics.

CHAPTER 12. REFERENCES

Adams G.P., Nasser, L.N., Bo, G.A., Garcia, A., Del Campo M.R., Mapletoft R.J., 1994a. Superovulatory response of ovarian follicles of Wave 1 versus Wave 2. Theriogenology 42, 1103-1113.

Adams, G.P., 1994. Control of ovarian follicular wave dynamics in cattle: Implications for synchronization & superstimulation. Theriogenology 41,19-24.

Adams, G.P., 1999. Comparative patterns of follicle development and selection in ruminants. J. Reprod. Fertil. (Suppl) 54,17–32.

Adams, G.P., Evans, A.C.O., Rawlings, N.C., 1994b. Follicular waves and circulating gonadotrophins in 8-month-old prepubertal heifers. J. Reprod. Fertil. 100, 27-33.

Adams, G.P., Kot, K., Smith, C.A., Ginther, O.J., 1993a. Selection of a dominant follicle and suppression of follicular growth in heifers. Anim. Reprod. Sci. 30,259-271.

Adams, G.P., Kot, K., Smith, C.A., Ginther, O.J., 1993b. Effect of the dominant follicle on regression of its subordinates in heifers. Can. J. Anim. Sci. 73,267-275.

Adams, G.P., Jaiswal, R., Singh, J., Malhi, P., 2008. Progress in understanding ovarian follicular dynamics in cattle. Theriogenology 69,72-80.

Adams, G.P., Matteri, R.L., Ginther, O.J., 1992a. Effect of progesterone on ovarian follicles, emergence of follicular waves and circulating follicle-stimulating hormone in heifers. J. Reprod. Fertil. 96,627–640.

Adams, G.P., Matteri, R.L., Kastelic, J.P., Ko, J.C.H., Ginther, O.J. 1992b. Association between surges of follicle stimulating hormone and the emergence of follicular waves in heifers. J. Reprod. Fertil. 94:177-188.

Adams, G.P., McCorkell, R.B., Jurgielewicz, V.C., Ambati, D., Woodbury, M.R., 2010. Estrous synchronization and fixed-time AI in wood bison (*Bison bison athabascae*). Reprod. Fertil. Dev. 22(1),255.

Adams, G.P., Pierson, R.A., 1995. Bovine model for study of ovarian follicular dynamics in humans. Theriogenology 43,113-120.

Adams, G.P., Singh, J., 2015. Ovarian follicular and luteal dynamics in cattle. In: John Wiley & Sons, Inc. (Eds), *Bovine Reproduction*, Ed. Wiley Blackwell, p. 219-244.

Allen, W.R., 1969. The immunological measurement of pregnant mare serum gonadotrophin. J. Endocrinology 32, 593–598.

Allen, W.R., Hamilton, D.W., Moor, R.M., 1973. The origin of equine endometrial cups II Invasion of the endometrium by trophoblast. Anat. Rec. 177,485–502.

Allen, W.R., Moor, R.M., 1972. The origin of the equine endometrial cups. I. Production of PMSG by fetal trophoblast cells. J. Reprod. Fertil. 29,313-316.

Alvarez, R.H., Martinez, A.C., Pires, R.M., 2010. Superovulatory response of zebu cows treated with pFSH in a singles subcutaneous injection followed by an additional intramuscular sub-dose 48 h later. Reprod. Dom. Anim. 45,421-424.

Amstalden, M., Williams, G.L., 2015. Neuroendocrine Control of Estrus and Ovulation. In: John Wiley & Sons, Inc. (Eds), *Bovine Reproduction*, Ed. Wiley Blackwell, p. 203-218.

Andersen, P.K., Geskus, R.B., de Witte, T., Putter, H., 2012. Competing risks in epidemiology: possibilities and pitfalls. Int. J. Epidemiol. 41,861-870.

Andersen, C. Y., Ezcurra, D., 2014. Human steroidogenesis: implications for controlled ovarian stimulation with exogenous gonadotropins. Reprod. Biol. Endocrinol. 12,128.

Aparicio, I.M., Garcia-Herreros, M., O'shea, L.C., Hensey, C., Lonergan, P., Fair, T., 2011. Expression, regulation, and function of progesterone receptors in bovine cumulus oocyte complexes during in vitro maturation. Biol. Reprod. 84,910-921.

Arreguin-Arevalo, J.A., Davis, T.L., Nett, T.M., 2007. Differential modulation of gonadotropin secretion by selective estrogen receptor 1 and estrogen receptor 2 agonists in ovariectomized ewes. Biol. Reprod. 77,320–328.

Ascoli, M., Fanelli, F., Segaloff, D.L., 2002. The lutropin/choriogonadotropin receptor, a 2002 perspective. Endocr Rev 23(2),141–174.

Azawi, O.I., Al-Mola, M.K.M., 2010. A study on superovulation using FSH and eCG in Awassi ewes. Trop. Anim. Health Prod. 42, 799–801.

Baruselli, P.S., Madureira, E.H., Visintin, J.A., Porto-Filho, R., Carvalho, G., Campanile, N.A.T., Zicarelli, Z., 2000. Failure of oocyte entry into oviduct in superovulated buffalo. Theriogenology 53:491.

Baruselli, P.S., Marques, M.O., Arruda, R.P., Carvalho, N.A.T., Oliveira, C.A., 2002. Plasma estradiol and progesterone concentrations in superovulate buffalo in presence of CIDR-B devices. Theriogenology 57:761.

Baruselli, P.S., Soares, J.G., Gimenes, L.U., Monteiro, B.M., Olazarri, M.J., Carvalho, N.A.T., 2013. Control of buffalo follicular dynamics for artificial insemination, superovulation and in vitro embryo production. Buffalo Bulletin 32(Special Issue 1),160-176.

Baracaldo, M.I., Martinez, M., Adams, G.P., Mapletoft, R.J., 2000. Superovulatory response following transvaginal follicle ablation in cattle. Theriogenology 53,1239-1250.

Baril, G., Vallet, J.C., 1990. Time of ovulations in dairy goats induced to superovulate with porcine follicle stimulating hormone during and out of the breeding season. Theriogenology, 34,303-311.

Barrett, D.M.W., Bartlewski, P.M., Batista-Arteaga, M., Symington, A., Rawlings, N.C., 2004. Ultrasound and endocrine evaluation of the ovarian response to a single dose of 500 IU of eCG following a 12-day treatment with progestogen-releasing intravaginal sponges in the breeding and nonbreeding seasons in ewes. Theriogenology 61,311-327.

Barrios, D.R., Kraemer, D.C., Bessoudo, E., Adams, L.G., 1988. Failure to isolate Brucella abortus from embryos or ova from culture-positive superovulated cows. Theriogenology 29,353-361.

Barros, C.M, Barcelos, A.C.Z, Gouvêa, L.M., Meneghel, M., Barcelos, D.S., Barcelos, L.N., Trinca, L.A., 2007. Improvement of a superstimulatory protocol in nelore cows: Replacing the last two doses of pFSH by eCG. Reprod Fertil Dev 20(1),152.

Baruselli, P.S., Ferreira, R.M., Salesa, J.N.S., Gimenes, L.U., Sá Filho, M.F., Martins, C.M., Rodrigues, C.A., Bo, G.A., 2011. Timed embryo transfer programs for management of donor and recipient cattle. Theriogenology 76,1583–1593.

Baruselli, P.S., Mucciolo, R.G., Arruda, R., Madureira, E.H., Amaral, R., Assumpcao, M.E.O.A., 1999. Embryo recovery rate in superovulated buffalo. Theriogenology 51,401.

Bazer, F.W., Ott, T.L., Spencer, T.E., 1998. Maternal recognition of pregnancy: comparative aspects. Troph. Res. 12,375-386.

Beckett, F.W., MacDiarmid, S.C., 1985. The effect of reduced-dose Brucella abortus strain 19 vaccination in accredited dairy herds. Br. Vet. J. 14,507–514

Ben-David, A., Davidson, C.E., 2014. Estimation method for serial dilution experiments. J. Microbiol. Methods 107,214-221.

Bergfelt, D.R., Bo, G.A., Mapletoft, R.J., Adams, G.P., 1997. Superovulatory response following ablation-induced synchrony of follicular wave emergence at random stages of the oestrous cycle in cattle. Anim. Reprod. Sci. 49,1-12.

Bergfelt, D.R., Lightfoot, K.C., Adams, G.P., 1994. Ovarian synchronization following ultrasound-guided transvaginal follicle ablation in heifers. Theriogenology 42,895-907.

Bernal, J. 2002. Action of thyroid hormone in brain. J. Endocrinol. Invest. 25:268–288.

Bernues, A., Manrique, E., Maza, M.T., 1997. Economic evaluation of bovine brucellosis and tuberculosis eradication programmes in a mountain area of Spain. Prev. Vet. Med. 30,137-149.

Bielanski, A., 2007. Disinfection procedures for controlling microorganisms in the semen and embryos of humans and farm animals. Theriogenology 68,1-22.

Bielanski, A., Bergeron, H., Lau, P.C K., Devenish, J., 2003. Microbial contamination of embryos and semen during long term banking in liquid nitrogen. Cryobiology 46, 146-152.

Bielanski, A., Dubuc, C., 1994. In vitro fertilization and culture of ova from heifers infected with bovine herpesvirus-1 (BHV-1). Theriogenology 41,1211-1217.

Bielanski, A., Stewart, B., 1996. Ubiquitous microbes isolated from in vitro fertilization (IVF) system. Theriogenology 1,269.

Blondin, P., Sirard, M.A., 1995. Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. Mol. Reprod. Dev. 41,54-62.

Blyth, C.B., 1995. Dynamics of ungulate populations in Elk Island National Park. M.Sc. Thesis. University of Alberta, Edmonton, AB.

Bo, G.A., Adams, G.P., Caccia, M., Martinez, M., Pierson, R.A., Mapletoft, R.J., 1995b. Ovarian follicular wave emergence after treatment with progestogen and estradiol in cattle. Anim. Reprod. Sci. 39,193-204.

Bo, G.A., Adams, G.P., Pierson, R.A., Mapletoft, R.J., 1995a. Exogenous control of follicular wave emergence in cattle. Theriogenology 43,31-40.

Bo, G.A., Adams, G.P., Pierson, R.A., Tribulo, H.E., Caccia, M., Mapletoft, R.J., 1994a. Follicular wave dynamics after estradiol-17β treatment of heifers with or without a progestogen implant. Theriogenology 41,1555-1569.

Bo, G.A., Baruselli, P.S., Chesta, P.M., Martins, C.M., 2006. The timing of ovulation and insemination schedules in superstimulated cattle. Theriogenology 65,89–101.

Bo, G.A., Baruselli, P.S., Moreno, D., Cutaia, L., Caccia, M., Tribulo, R., Tribulo, H., Mapletoft, R.J., 2002. The control of follicular wave development for self-appointed embryo transfer programs in cattle. Theriogenology 57,53-72.

Bo, G.A., Guerrero, D.C., Adams, G.P., 2008. Alternative approaches to setting up donor cows for superstimulation. Theriogenology 69,81-87.

Bo, G.A., Guerrero, D.C., Tríbulo, A., Tríbulo, H., Tríbulo, R., Rogan, D., Mapletoft, R.J., 2010. New approaches to superovulation in the cow. Reprod. Fert. Dev. 22,106-112.

Bo, G.A., Hockley, D.K., Nasser, L.F., Mapletoft, R.J., 1994b. Superovulatory response to a single subcutaneous injection of Folltropin V in beef cattle. Theriogenology 42,963-975.

Bo, G.A., Mapletoft, R.J., 2014. Historical perspectives and recent research on superovulation in cattle. Theriogenology, 81,38-48.

Bodensteiner, K.J., Wiltbank, M.C., Bergfelt, D.R., Ginther, O.J., 1996. Alterations in follicular estradiol and gonadotropin receptors during development of bovine antral follicles. Theriogenology 45,499–512.

Boland, M.P., Crosby, T.F., Gordon, I., 1978. Morphological normality of cattle embryos following superovulation using PMSG. Thetiogenology 10,175-180.

Booth, P.J., Stevens, D.A., Collins, M. E., Brownlie, J., 1995. Detection of bovine viral diarrhoea virus antigen and RNA in oviduct and granulosa cells of persistently infected cattle. J. Reprod. Fertil. 105,17-24.

Bossi, P., Tegnell, A., Baka, A., Van Loock, F., Hendriks, J., Werner, A., Maidhof, H., Gouvras, G., 2004. Bichat guidelines for the clinical management of brucellosis and bioterrorism-related brucellosis. Euro. Surveill. 9,E15-E16.

Brownlie, J., Booth, P.J., Stevens, D.A., Collins, M.E., 1997. Expression of non-cytopathogenic bovine viral diarrhoea virus (BVDV) in oocytes and follicles of persistently infected cattle. Vet. Rec. 141,335-337.

Bundle, D.R., Cherwonogrodzky, J.W., Caroff, M., Perry, M.B., 1987. The lipopolysaccharides of Brucella abortus and B. melitensis. Annales de l'Institut Pasteur/Microbiologie 138,92-98.

Canadian Food Inspection Agency, 1999. Risk assessment on bovine brucellosis and tuberculosis in Wood Buffalo National Park and area. Animal, Plant and Food Risk Analysis Network, Ottawa, Ontario, Canada. 38 pp.

Canadian Food Inspection Agency, 2015. Fact Sheet-Brucellosis. How does the Canadian Food Inspection Agency (CFIA) maintain Canada's brucellosis-free status for livestock? http://www.inspection.gc.ca/animals/terrestrial-animals/diseases/reportable/brucellosis/fact-sheet/eng/1305673222206/1305673334337. Accessed on August 2015.

Carbyn, L.N., Lunn, N.J., Timoney, K., 1998. Trends in the distribution and abundance of bison in Wood Buffalo National Park. Wildl. Soc. Bull. 26,463–470

Casarini, L., Lispi, M., Longobardi, S., Milosa, F., La Marca, A., Tagliasacchi, D., Pignatti, E., Simoni, M., 2012. LH and hCG action on the same receptor results in quantitatively and qualitatively different intracellular signalling. PLoS ONE 7(10),e46682.

Cervantes, M.P., Anzar, M., Mapletoft, R.J., Palomino, J.M., Adams, G.P., 2013. Nuclear maturation of wood bison (*Bison bison athabascae*) cumulus–oocyte complexes. Reprod. Fertil. Dev. 25,283-283.

Cervantes, M.P., Palomino, J.M., Anzar, M., Mapletoft, R.J., Mastromonaco, G., Adams, G.P., 2015. *In vitro* embryo production using in vivo-matured oocytes collected transvaginally from wood bison (*Bison bison athabascae*). Reprod. Fertil. Dev. 27,213-214.

Cheng, K.W., Leung, P.C.K., 2000. The expression, regulation and signal transduction pathways of the mammalian gonadotropin releasing hormone receptor. Can. J Physiol. Pharmacol. 78,1029–1052.

Cheville, N.F., McCullough, D.R., Paulson, L.R., 1998. Brucellosis in the greater Yellowstone area. Washington D.C., USA, National Academy Press. 186 pp.

Cheville, N.F., Stevens, M.G., Jensen, A.E., Tatum, F.M., Halling, S.M., 1993. Immune responses and protection against infection and abortion in cattle experimentally vaccinated with mutant strains of Brucella abortus. Am. J. Vet. Res. 54,1591–1597.

Choi, J., Smitz, J., 2014. Luteinizing hormone and human chorionic gonadotropin: origins of difference. Mol. Cell. Endocrinol. 383,203-213.

Chrousos, G.P., 1998. Stressors, stress, and neuroendocrine integration of the adaptive response. The 1997 Hans Selye Memorial Lecture. Ann. NY Acad. Sci. 851,311–35.

Cifuentes, E., Quevedo, L., Hoyos, A., Carballo, D., Piccardi, M., Bó, G.A., 2009. Efecto de la aplicacion de eCG en vacas donantes de embriones superovuladas con Folltropin-V. In: Proceedings of the 8vo Simposio Internacional de Reproduccion Animal , IRAC, Córdoba, Argentina. [Abstract].

Clapp, B., Walters, N., Thornburg, T., Hoyt, T., Yang, X., Pascual, D.W., 2011. DNA vaccination of bison to brucellar antigens elicits elevated antibody and IFN-γ responses. J. Wildl. Dis. 47,501-510.

Cognie, Y., Baril, G., Poulin, N., Mermillod, P., 2003. Current status of embryo technologies in sheep and goat. Theriogenology 59,171-188.

Colazo, M. G., Gordon, M. B., Rajamahendran, R., Mapletoft, R. J., Ambrose, D. J., 2009. Pregnancy rates to timed artificial insemination in dairy cows treated with gonadotropin-releasing hormone or porcine luteinizing hormone. Theriogenology 72, 262–270.

Cole, L.A., 2010. Biological functions of hCG and hCG-related molecules. Reprod. Biol. Endocrinol. 8,102

Comizzoli. P., Mermillod, P., Mauget, R., 2000. Reproductive biotechnologies for endangered mammalian species. Reprod. Nutr. Dev. 40,493-504.

Corbel, M.J., 1997. Brucellosis: an overview. Emerg. Infect. Dis. 3,213-221.

Corner, A.H., Connell, R., 1958. Brucellosis in bison, elk, and moose in Elk Island National Park, Alberta, Canada. Can. J. Comp. Med. Vet. Sci. 22,9-21.

COSEWIC, 2013. COSEWIC assessment and status report on the Plains Bison *Bison bison bison* and the Wood Bison *Bison bison athabascae* in Canada. Committee on the Status of Endangered Wildlife in Canada. Ottawa.109 pp.

D'Occhio, M.J., Sudha, G., Jillella, D., Whyte, T., Maclellan, L.J., Walsh, J., Trigg, T.E., Miller, D., 1997. Use of a GnRH agonist to prevent the endogenous LH surge and injection of exogenous LH to induce ovulation in heifers superstimulated with FSH: a new model for superovulation. Theriogenology 47,601–613.

Dahl, G. E., Buchanan, B. A., Tucker, H. A., 2000. Photoperiodic effects on dairy cattle: A review. J Dairy Sci. 83,885–893.

Dahlen, C.R., Marquezini, G.H.L., Larson, J.E., Lamb, G.C. 2011. Fixed-time artificial insemination in replacement beef heifers after estrus synchronization with human chorionic gonadotropin or gonadotropin-releasing hormone. J. Anim. Sci. 89,2750-2758.

Davis, D.S., 1990. Brucellosis in wildlife. In K. Neilsen & J.R. Duncan (Eds.) *Animal brucellosis*. CRC Press, Boca Raton, Florida, p. 322–334.

Davis, D.S., Elzer, P., 1999. Safety and efficacy of *Brucella abortus* RB51 vaccine in adult American bison. Proc. US Anim. Health As. 103,154-158.

Davis, D.S., Templeton, J.W., Ficht, T.A., Huber, J.D., Angus, R.D., Adams, L.G., 1991. *Brucella abortus* in bison. II. Evaluation of strain 19 vaccination of pregnant cows. J. Wildl. Dis. 27,258-264.

Davis, R.L., Arteaga, A., Hasler, J.F., 2011. Addition of equine chorionic gonadotropin to a traditional follicle stimulating hormone protocol for superovulation of bos taurus beef cows. Reprod. Fertil. Dev. 24(1),224-225.

De Rensis, F., F. Lopez-Gatius, I. Garcia-Ispierta, and M. Techakumpu. 2010. Clinical use of human chorionic gonadotropin in dairy cows: An update. Theriogenology 73:1001–1008.

Del Campo, M.R., Tamayo, R., Del Campo, C.H., 1987. Embryo transfer from brucellosis-positive donors: a field trial. Theriogenology 27, 221.

Dias, F.C.F., Costa, E., Adams, G.P., Mapletoft, R.J., Kastelic, J., Dochi, O., Singh, J., 2013a. Effect of duration of the growing phase of ovulatory follicles on oocyte competence in superstimulated cattle. Reprod. Fertil. Dev. 25,523-530.

Dias, F.C.F., Dadarwal, D., Adams, G.P., Mrigank, H., Mapletoft, R.J., Singh, J., 2013b. Length of the follicular growing phase and oocyte competence in beef heifers. Theriogenology 79, 1177–1183.

Diaz, T., Schmitt, E.J., De la Sota, R.L., Thatcher, M.J., Thatcher, W.W., 1998. Human chorionic gonadotropin-induced alterations in ovarian follicular dynamics during the estrous cycle of heifers. J. Anim. Sci. 76, 1929-1936.

Donadeu, F.X., Watson, E.D., 2007. Seasonal changes in ovarian activity: Lessons learnt from the horse. Anim. Reprod. Sci. 100,225-242.

Dorn, C.G., 1995. Application of reproductive technologies in North American bison (Bison bison). Theriogenology, 43,13–20.

Dorn, C.G., Fosworth, W.B., Butler, P.D., Olsen, G.C., Wolfe, B.A., Davis, D.S., Simpson, T.R., Kraemer, D.C., 1990. Superovulation and embryo recovery in the american bison (*Bison bison*). Theriogenology 33, 217.

Driancourt, M.A., 2001. Regulation of ovarian follicular dynamics in farm animals. Implications for manipulation of reproduction. Theriogenology 55,1211-1239.

Ebel, E.D., Williams, M.S., Tomlinson, S.M., 2008. Estimating herd prevalence of bovine brucellosis in 46 USA states using slaughter surveillance. Prev. Vet. Med. 85,295–316.

Edqvist, L. 1993. The endogenous control of reproductive processes. In: G.J. King (Eds) *Reproduction in domestic animals*. Elsevier Science Publishers B.V. p. 75-81.

Elsden, R.P., Hasler, J.F., Seidel, G.E., 1976. Non-surgical recovery of bovine eggs. Theriogenology 6,523-532.

Environmental Assessment Panel, 1990. Northern diseased bison. Report of the Environmental Assessment Panel. Fed. Env. Assess. Rev. Office. pp 47.

Evans, A.C.O., Adams, G.P., Rawlings, N.C., 1994. Endocrine and ovarian changes leading up to the first ovulation in prepubertal heifers. J. Reprod. Fertil. 100, 187-194.

Faiman, C., Ryan, R.J., Zwirek, S.J., Rubin, M.E., 1968. Serum FSH and HCG during human pregnancy and puerperium. J. Clin. Endocrinol. Metab. 28,1323-1329.

Farin, C.E., Moeller, C.L., Mayan, H., Gamboni, F., Sawyer, H.R., Niswender, G.D., 1988. Effect of luteinizing hormone and human chorionic gonadotropin on cell populations in the ovine corpus luteum. Biol. Reprod. 38,413–421.

Filicori, M., Fazleabas, A.t., Huhtaniemi, I., Licht, P., Rao, C.V., Tesarik, J., Zygmunt, M., 2005. Novel concepts of human chorionic gonadotropin: reproductive system interactions and potential in the management of infertility. Fertil. Steril.84, 275-284.

Fink, G. 2000. Neuroendocrine regulation of pituitary function: general principles. In: P.M. Conn, M.E. Freeman (Eds) *Neuroendocrinology in physiology and medicine.* Totowa, NJ, USA, Humana Press. p. 107–134.

Forbes, L.B., 1980. The eradication of bovine brucellosis: an effective sample acquisition protocol for a low incidence area. Proc 23rd Annual Meeting American Association Vet Lab Diagnostics. Pages 195-216.

Fortune, J.E., 1994. Ovarian follicular growth and development in mammals. Biol. Reprod. 50,225–32.

Frankham R. 2005. Genetics and extinction. Biol Conserv. 126:131–140.

Fuller, W.A., 1966. The ecology and management of bison in Wood Buffalo National Park. Canadian Wildlife Service. Otawa, Canada.Wildlife Management Bulletin Series 1, 16,1-52.

Galet, C., Ascoli, M., 2005. The differential binding affinities of the luteinizing hormone (LH)/choriogonadotropin receptor for LH and choriogonadotropin are dictated by different extracellular domain residues. Mol. Endocrinol. 19,1263-1276.

Gall, D., Nielsen, K., Forbes, L., Davis, D., Elzer, P., Olsen, S., Balsevicius, S., Kelly, L., Smith, P., Tan, S., Joly, D., 2000. Validation of the fluorescence polarization assay and comparison to other serological assays for the detection of serum antibodies to Brucella abortus in bison. J. Wildl. Dis. 36,469-476.

Gallien, P., Dorn, C., Alban, G., Staak, C., Protz, D., 1998. Detection of Brucella species in organs of naturally infected cattle by polymerase chain reaction. Vet. Rec. 142,512-514.

García Guerra, A., Tribulo, A., Yapura, J., Adams, G. P., Singh, J., Mapletoft, R. J., 2015. Lengthened superstimulatory treatment in cattle: Evidence for rescue of follicles within a wave rather than continuous recruitment of new follicles. Theriogenology 84,467–476.

García Guerra, A., Tribulo, A., Yapura, J., Singh, J., Mapletoft, R. J., 2012. Lengthening the superstimulatory treatment protocol increases ovarian response and number of transferable embryos in beef cows. Theriogenology, 78,353-360.

Garret, J.J. 2007. A triadic relationship on the northern Great Plains: Bison (Bison bison), native plants, and native people. PhD thesis. Department of Forest, Rangeland, Watershed Stewardship, Fort Collins, Colorado State University.

Garry, F., 2008. Miscellaneous infectious diseases. In: T.J. Divers and S.F. Peek (Eds) *Rebhun's Diseases of dairy cattle, Second edition.* Phildelphia, PA, USA, Saunders. p. 606-639.

Gates, C.C., Chowns, T., Reynolds, H., 1992. Wood buffalo at the crossroads. Alberta: studies in the arts and sciences 3, 139-165.

Gates, C.C., Freese, C.H., Gogan, P.J.P. Kotzman, M., 2010. American Bison: Status Survey and Conservation Guidelines 2010. Gland, Switzerland: IUCN, 134 pp.

Gates, C.C., Stephenson, R.O., Reynolds, H.W., van Zill de Jong, C.G., Schwantje, H., Hoefs, M., Nishi, J., Cool, N., Chisholm, J., James, A., Koonz, B., 2001. National recovery plan for the wood bison (Bison bison athabascae). National Recovery Plan No 21. Recovery of Endangered Wildlife (RENEW). Ottawa, Ontario, Canada. 50 pp.

Geist, V., 1991. Phantom subspecies: the wood bison Bison bison" athabascae" Rhoads 1897 is not a valid taxon, but an ecotype. Arctic, 44,283-300.

Ginther, O.J., Beg, M.A., Bergfelt, D.R., Donadeu, F.X., Kot, K., 2001a. Follicle selection in monovular species. Biol. Reprod. 65,638–647.

Ginther, O.J., Bergfelt, D.R., 1990. Effect of GnRH treatment during the anovulatory season on multiple ovulation rate and on follicular development during the ensuing pregnancy in mares. J. Reprod. Fertil. 88, 119–126.

Ginther, O.J., Bergfelt, D.R., Beg, M.A., Kot, K., 2001b. Follicle selection in cattle: role of luteinizing hormone. Biol. Reprod. 64,197-205.

Ginther, O.J., Bergfelt, D.R., Kulick, L.J., Kot, K., 1999. Selection of the dominant follicle in cattle: establishment of follicle deviation in less than 8 hours through depression of FSH concentrations. Theriogenology 52,1079-1093.

Ginther, O.J., Bergfelt, D.R., Kulick, L.J., Kot, K., 2000. Selection of the dominant follicle in cattle: role of estradiol. Biol. Reprod. 63,383-389.

Ginther, O.J., Kastelic, J.P., Knopf, L. 1989. Composition and characteristics of follicular waves during the bovine estrous cycle. Anim. Reprod. Sci. 20, 187-200.

Ginther, O.J., Kot, K., Kulick, L.J., Wiltbank, M.C., 1997. Emergence and deviation of follicles during the development of follicular waves in cattle. Theriogenology 48,75–87.

Ginther, O.J., Wiltbank, M.C., Fricke, P.M., Gibbons, J.R., Kot, K., 1996. Selection of the dominant follicle in cattle. Biol. Reprod. 55,1187-1194.

Givens, M.D., Gard, J.A., Stringfellow, D.A., 2007. Relative risks and approaches to biosecurity in the use of embryo technologies in livestock. Theriogenology 68,298-307.

Givens, M.D., Marley, S.D., 2008. Approaches to biosecurity in bovine embryo transfer programs. Theriogenology 69,129-136.

Gonzalez, A., Lussier, J.G., Carruthers T.D., Murphy, B.D., Mapletoft, R.J., 1990. Superovulation of beef heifers with Folltropin. A new FSH preparation containing reduced LH activity, Theriogenology 33,519–529.

González, F., Calero, P., Beckers, J.F., 2002. Induction of superovulation in domestic ruminants. Biotech. Anim. Husbandry 5,209-223.

Goodrowe, K.L., Mastromonaco, G.F., Othen, L.S. 2007. Reproductive patterns in female bison (*Bison bison sp.*). In: R.S. Youngquist, W.R. Threlfall (Eds) *Current therapy in large animal theriogenology, Second edition*. St. Louis, Missouri, USA, Saunders. p. 1000-1004.

Gordon, I.R., 2004. Reproductive technologies in farm animals. Cambridge, MA, USA, CABI Publishing. 332 pg.

Gordon, M.B. 2007. Strategies to improve ovulation synchronization/timed artificial insemination protocol to increase pregnancy rate in dairy cattle. PhD thesis. Faculty of Graduate Studies, Vancouver, University of British Columbia.

Gordon, M.B., 2011. Strategies to improve ovulation synchronization/timed artificial insemination protocol to increase pregnancy rate in dairy cattle. PhD thesis. Faculty of Graduate Studies, Vancouver, University of British Columbia.

Goto, K., Nakanishi, Y., Ohkutsu, S., Ogawa, K., Tasaki, M., Ohta, M., Inohae, S., Tateyama, S., Kawabata, T., 1987. Plasma progesterone profiles and embryo quality in superovulated Japanese black cattle. Theriogenology, 27,819-826.

Goto, K., Ohkot, S, Nakanishi, Y., Ogawa, K., Tasaki, M., Inohae, S., Tateyama, S., Kawabata, T., Ishii S., Miyamoto A., Furusawa, T., Umezu M., Masaki, J., 1988. Endocrine profiles and embryo quality in superovulated Japanese Black Cattle. Theriogenology 29,615–629.

Goulding, D., Williams, D.H., Rochel, J.F., Bolands, M.P., 1991. Superovulation in heifers using either pregnant mare serum gonadoptrophin or follicle stimulating hormone during the mid luteal stage of the estrous cycle. Theriogenology 36,949-958.

Goulding, D., Williams, D.H., Rochel, J.F., Bolands, M.P., 1996. Factors affecting superovulation in heifers treated with PMSG. Theriogenology 45,765-773.

Green, W.C.H., Rothstein, A., 1991. Trade-offs between growth and reproduction in female bison. Oecologia 86,521-527.

Greve, T., Callesen, H., Hyttel, P., Høier, R., Assey, R., 1995. The effects of exogenous gonadotropins on oocyte and embryo quality in cattle. Theriogenology, 43,41-50.

Greve, T., Lehn-Jensen, H., Rasbech, N.O., 1977. Non-surgical recovery of bovine embryos. Theriogenology 7,239-250.

Gündoğan, M., Baki, D., Yeni, D., 2003. Reproductive Seasonality in Sheep. Acta Agric. Scan. Sec. A Anim. Sci. 53,175-179.

Habib, K.E., Gold, P.W., Chrousos, G.P., 2001. Neuroendocrinology of stress. Endocrinol. Metab. Clin. North Am. 30,695–728.

Haigh, J.C., Berezowski, J., Munger, R., 2000. Reproduction and herd health. In: Proceeding of the Second International Bison Conference, Edmonton, AB, Canada, 1-4 August, 2000. p. 154-173.

Hansen, P., 2014. Current and future assisted reproductive technologies for mammalian farm animals. In: G.C. Lamb and N. DiLorenzo (eds.), Current and Future Reproductive Technologies and World Food Production, Advances in Experimental Medicine and Biology. Springer Science Business Media New York, USA. 752,1-12.

Hartop, B.V., Mandeville, W., Lee, N., Johnson, D., 2009. Bison Control Area Program Annual Report of Survey Activities November 2007–April 2008. Department of Environment and Natural Resources, Government of the Northwest Territories. Manuscript Report N^{o} 207, 55pp.

Hazlerigg, D.G., Morgan, P.J., Messager, S., 2001. Decoding photoperiodic time and melatonin in mammals: What can we learn from the pars tuberalis? J. Biol Rhythms. 16,326-335.

Hedrick, P.W., 2009. Conservation genetics and North American bison (Bison bison). J. Heredity 100,411-420.

Helbig, L., Woodbury M.R., Haigh, J.C., Barth, A.D., 2007. The onset of puberty in North American bison (*Bison bison*) bulls. Anim. Reprod. Sci. 97,12-24.

Himsworth, C.G., Elkin, B.T., Nishi, J.S., Neimanis, A.S., Wobeser, G.A., Turcotte, C., Leighton, F.A., 2010. An outbreak of bovine tuberculosis in an intensively managed conservation herd of wild bison in the Northwest Territories. Can. Vet. J. 51, 593-597.

Holt, W.W., Pickard, A.R., 1999. Role of reproductive technologies and genetic resource banks in animal conservation. Rev. Reprod. 4,143–150.

Humblot, P., Holm, P., Lonergan, P., Wrenzycki, C., Lequarré, A. S., Joly, C. G., Hermann, D., Lopes, A., Rizos, D., Niemann, H., Callesen, H., 2005. Effect of stage of follicular growth during superovulation on developmental competence of bovine oocytes. Theriogenology 63,1149-1166.

Iida, K., Kobayashi, N., Kohno, H., Miyamoto, A., Fukui, Y., 2004. A comparative study of induction of estrus and ovulation by three different intravaginal devices in ewes during the non-breeding season. J. Reprod. Dev. 50,63-69.

Inskeep, E.K., 2004. Preovulatory, postovulatory, and postmaternal recognition effects of concentration of progesterone on embryonic survival in the cow. J. Anim. Sci. 82(E. Suppl.), E24-E39.

Jacques, I., Grayon, M., Berthelot, F., Garin-Bastuji, B., Guilloteau, L., Martinat-Botté, F., 2004. Risk of Brucella transmission by porcine embryos: an in vitro study. In: Proceedings of the Congress of the International Society of Animal Hygiene, October 11 -13, 2004, Saint-Malo, France 1,129 - 130.

Jamnongjit, M., Hammes, S.R., 2006. Ovarian steroids: the good, the bad, and the signals that raise them. Cell cycle 5,1178-1183.

Joly, D.O., Messier, F. 2001. Limiting effects of bovine brucellosis and tuberculosis on bison within Wood Buffalo National Park. Final report. University of Saskatchewan.

Joly, D.O., Messier, F. 2004b. Factors affecting apparent prevalence of tuberculosis and brucellosis in wood bison. J. Anim. Ecol. 73:623-663.

Joly, D.O., Messier, F., 2004a. Testing hypotheses of bison population decline (1970–1999) in Wood Buffalo National Park: synergism between exotic disease and predation. Can. J. Zool. 82,1165–1176.

Joly, D.O., Messier, F., 2005. The effect of bovine tuberculosis and brucellosis on reproduction and survival of wood bison in wood buffalo national park. J. Anim. Ecol. 74,543–551.

Kafi, M., McGowan, M.R., 1997. Factors associated with variation in the superovulatory response of cattle. Anim. Reprod. Sci. 48,137-157.

Karsch, F.J., Bittman, E.L., Foster, D.L., Goodman,R.L., Legan, S.J., Robinson, J.E., 1984. Neuroendocrine basis of seasonal reproduction. Recent Prog. Horm. Res. 40,185-232.

Karsch, F.J., Bowen, J.M., Caraty, A., Evans, N.P., Moenter, S.M., 1997. Gonadotropin-releasing hormone requirements for ovulation. Biol. Reprod. 56,303-309.

Karsch, F.J., Dahl, G.E., Evans, N.P., Manning, J.M., Mayfield, K.P., Moenter, S.M., Foster, D.L., 1993. Seasonal changes in GnRH secretion in ewe: alteration in response to the negative feedback action of estradiol. Biol. Reprod. 49,1377–1383.

Kinder, J.E., Bergfeld, E.G., Wehrman, M.E., Peters, K.E., Kojima, F.N., 1994. Endocrine basis for puberty in heifers and ewes. J. Reprod. Fertil. Sup. 49,393-407.

Kinder, J.E., Kojima, F.N., Bergfeld, E.G., Wehrman, M.E., Fike, K.E., 1996. Progestin and estrogen regulation of pulsatile LH release and development of persistent ovarian follicles in cattle. J. Anim. Sci. 74,1424–1440.

Kirkpatrick, J.F., Kincy, V., Bancroft, K., Shideler, S.E., Lasley, B.L., 1991. Oestrous cycle of the North American bison (*Bison bison*) characterized by urinary pregnanediol-3-glucuronide, J. Reprod. Fertil. 93,541-547.

Kriegsfeld, L.J., Gibson, E.M., Williams III, W.P., Zhao, S., Mason, A.O., Bentley, G.E., Tsutsui, K., 2010. The roles of RFamide-related peptide-3 in mammalian reproductive function and behaviour. J. Neuroendocrinol. 22,692–700

Lamb, G.C., Dahlen, C.R., Larson, J.E., Marquezini, G., Stevenson, J.S., 2010. Control of the estrous cycle to improve fertility for fixed-time artificial insemination in beef cattle: A review. J. Anim. Sci. 88(E. Suppl.),E181–E192.

Larter, N.C., Gates, C.C., 1990. Home ranges of wood bison in an expanding population. J. Mammalogy 71,604-607.

Larter, N.C., Gates, C.C., 1991. Diet and habitat selection of wood bison in relation to seasonal changes in forage quantity and quality. Can. J. Zoolog. 69,2677-2685.

Larter, N.C., Sinclair, A.R.E., Ellsworth, T., Nishi, J., Gates, C.C., 2000. Dynamics of reintroduction in an indigenous large ungulate: the wood bison of northern Canada. Anim. Conserv. 3,299-309.

Laster, D.B., 1972. Disappearance of and uptake of [^{125}I] FSH in rat, rabbit, ewe and cow. J. Reprod. Fert. 30,407-415.

Legan, S.J. and Winans, S.S. 1981. The photoneuroendocrine control of seasonal breeding in the ewe. *Gen. Comp. Endocrinol.* 45:317-328.

Lessard, C., Danielson, J., Rajapaksha, K., Adams, G.P., McCorkell, R. 2009. Banking North American buffalo semen. Theriogenology 71, 1112-1119.

Levine, J.E., Chappel, P., Besecke, L.M., Bauer-Dantoin, A.C., Wolfe, A.M., Porkka-Heiskanen, T., Urban, J.H. 1995. Amplitude and frequency modulation of pulsatile luteinizing hormone-releasing hormone release. Cell. Mol. Neurobiol. 15:117–139.

Lincoln, G.A., Short, R.V., 1980. Seasonal breeding: nature's contraceptive. Recent Prog. Horm. Res. 36,1-52.

Lonergan, P., 2011. Influence of progesterone on oocyte quality and embryo development in cows. Theriogenology 76,1594–1601.

Lord, V.R., Schurig, G.G., Cherwonogrodzky, J.W., Marcano, M.J., Melendez, G.E., 1998. Field study of vaccination of cattle with Brucella abortus strains RB51 and 19 under high and low disease prevalence. Am. J. Vet. Res. 59,1016-1020.

Loskutoff, N.M., Bartel, P., Meintjes, M., Godke, R.A., Schiewe, M.C., 1995. Assisted reproductive technology in non domestic ungulates: a model approach to preserving and managing genetic diversity. Theriogenology 43,3-l 2.

Lovie, M., Garcia, A., Hackett, A., Mapletoft, R.J., 1994. The effect of dose schedule and route of administration on superovulatory response to folltropin in holstein cows. Theriogenology 41,241.

Lutze-Wallace, C., Turcotte, C., Stevenson, D.A., Elkin, B., Koller-Jones, M., Nishi, J., Wobeser, G., 2006. Isolation of Mycobacterium bovis from a wood bison in a wildlife conservation project in the Northwest Territories. Can. Vet. J., 47,317-318.

Madill, S., Rieger, D., Johnson, W.H., Walton, J.S., Coy, D.H., Rawlings, N.C. 1994. Effects of an LHRH antagonist on the time of occurrence and amplitude of the preovulatory LH surge, progesterone and estradiol secretion, and ovulation in superovulated Holstein heifers. Theriogenology 41,951-960.

Malpaux, B., 2000. The neuroendocrine control of seasonal rhytms. In: M. Coon, M. Freeman (Eds) *Neuroendocrinology in Physiology and Medicine.* New Jersey, USA, Humana Press. p. 435-451.

Malpaux, B., 2006. Seasonal regulations of reproduction in mammals. In: J.D. Neill (Eds) Knobil and Neill's Physiology of Reproduction. Third edition. St. Louis, MO, USA, Elsevier Academic Press publications. p. 2231-2281.

Malpaux, B., Migaud, M., Tricoire, H., and Chemineau, F., 2001. Biology of Mammalian Photoperiodism and the Critical Role of the Pineal Gland and Melatonin. J. Biol. Rhythms 16, 336-347.

Mann, G.E., La mming, G.E., 1999. The influence of progesterone during early pregnancy in cattle. Reprod Dom Anim 34,269 –274.

Mapletoft, R., 1986. Bovine embryo transfer. In: Current Therapy in Theriogenology 2. Morrow, D. A. (ed.), W. B. Saunders Co., Philadelphia, pp. 54-58.

Mapletoft, R.J., Bo, G.A., 2012. The evolution of improved and simplified superovulation protocols in cattle. Reprod. Fertil. Dev. 24, 278–283.

Mapletoft, R.J., Bo, G.A., 2015. Superovulation in cattle. In: John Wiley & Sons, Inc. (Eds), *Bovine Reproduction*, Ed. Wiley Blackwell, p. 696-702.

Mapletoft, R.J., Bó, G.A., Baruselli, P.S., 2009. Control of ovarian function for assisted reproductive technologies in cattle. Anim. Reprod. 6,114-124.

Mapletoft, R.J., Steward, K.B., Adams, G.P., 2002. Recent advances in the superovulation in cattle. Reprod. Nutr. Dev. 42,1–11.

Marquezini, G.H.L., Dahlen, C.R., Bird, S.L., Lamb, G.C., 2011. Administration of human chorionic gonadotropin to suckled beef cows before ovulation synchronization and fixed-time insemination: Replacement of gonadotropin-releasing hormone with human chorionic gonadotropin. J Anim. Sci. 89, 3030-3039.

Martinez, M.F., Adams, G.P., Bergfelt, D.R., Kastelic, J.P., Mapletoft, R.J., 1999. Effect of LH or GnRH on the dominant follicle of the first follicular wave in beef heifers. Anim. Reprod. Sci. 57, 23-33.

Martinuk, S.D., Manning, A.W., Black, W.D., Murphy, B.D., 1991. Effects of carbohydrates on the pharmacokinetics and biological activity of equine chorionic gonadotropin in vivo. Biol. Reprod. 45,598-604.

Matsuda, D.M., Bellem, A.C., Gartley, C.J., Madison, V., King, W.A., Liptrap, R.M., Goodrowe, K.L., 1996. Endocrine and behavioral events of estrous cyclicity and synchronization in wood bison (Bison bison athabascae). Theriogenology 45,1429–1441.

Mattos, M.C.C., Bastos, M.R., Guardieiro, M.M., Carvalho, J.O., Franco, M.M., Mourão, G.B., Barros, C.M., Sartori, R. 2011. Improvement of embryo production by the replacement of the last two doses of porcine follicle-stimulating hormone with equine chorionic gonadotropin in Sindhi donors. Anim. Reprod. Sci. 125,119– 123.

Matzuk, M.M., Hsueh, A.J.W., Lapolt, P., Tsafriri, A., Keene, J.L., Boime, I., 1990. The Biological Role of the Carboxyl-Terminal Extension of Human Chorionic Gonadotroin β-Subunit. Endocrinology 126, 376-383.

McCorkell, R.B., MacDougall, L., Adams, G.P., 2004. Ovarian follicle development in wapiti (*Cervus elaphus*) during the anovulatory season. Theriogenology 61,473–483.

McCorkell, R.B., Paziuk, W., Smart, L., Woodbury, M.R., Adams, G.P., 2010. Exogenous control of follicular wave emergence in wood bison (*Bison bison athabascae*). Reprod. Fertil. Dev. 22(1),257.

McCorkell, R.B., Woodbury, M.R., Adams, G.P., 2007. Ovarian follicular and luteal dynamics in wapiti during seasonal transitions. Theriogenology 67,1224–1232.

McCorkell, R.B., Woodbury, M.R., Adams, G.P., 2013a. Serial ovarian ultrasonography in wild-caught wood bison (Bison bison athabascae). Theriogenology. 80, 552-556.

McCorkell, R.B., Woodbury, M.R., Adams, G.P., 2013b. Superovulation in waptiti (*Cervus elaphus*) during the anovulatory season. Theriogenology 79, 24-27.

McCormack, P.A., 1992. The political economy of bison management in Wood Buffalo National Park. Arctic 45, 367-380.

McCracken, J.A., Custer, E.E., Lamsa, J.C., 1999. Luteolysis: a neuroendocrine-mediated event. Physiol. Rev. 79,264-324.

McFarlane, K., Wilson, G.A., Nishi, J.S., 2006. Management strategies for conservation of genetic diversity in wood bison *(Bison bison athabascae)*. University of Alberta. File report No 135.

McLeod, B.J., Meikle, L.M., Fisher, M.W., Shackell, G.H., Heath, D.A., 2001. Gonadotrophin-induced follicle development in red deer hinds during the breeding and non-breeding seasons. Reproduction 122, 111–119.

Menchaca, A., Vilariño, M., Crispo, M., De Castro, T., Rubianes, E., 2009. New approaches to superovulation and embryo transfer in small ruminants. Reprod. Fertil. Dev. 22,113-118.

Merton, J.S., De Roos, A.P.W., Mullaart, E., De Ruigh, L., Kaal, L., Vos, P.L.A.M., Dieleman, S.J., 2003. Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry. Theriogenology 59,651-674.

Mitchell, J.A., Gates, C.C., 2002. Status of the Wood Bison (*Bison bison athabascae*) in Alberta. Alberta Sustainable Resource Development, Fish and Wildlife Division, and Alberta Conservation Association, Wildlife Status Report No. 39, Edmonton, AB. p 32.

Mitchell, L.M., Dingwall, W.S., Mylne, M.J.A., Hunton, J., Matthews, K., Gebbie, F.E., McCallum, G.J., McEvoy, T.J., 2002. Season affects characteristics of the pre-ovulatory LH surge and embryo viability in superovulated ewes. Anim. Reprod. Sci. 74,163–174.

Monniaux, D., Chupin, D., Saumande, J., 1983. Superovulatory response of cattle. Theriogenology 19,55-81.

Moran, C., Quirke, J.F., Roche, J.F., 1989. Puberty in heifers: a review. Anim. Reprod. Sci. 18, 167-182.

Moriyon, I., Grillo, M.J., Monreal, D., Gonzalez, D., Marin, C., Lopez-Goni, I., Mainar-Jaime, R.C., Moreno, E., Blasco, J.M., 2004. Rough vaccines in animal brucellosis: structural and genetic basis and present status. Vet. Res. 35,1–38.

Mumford, E.L., Squires, E.L., Peterson, K.D., Nett, T.M., Jasko, D.J. 1994. Effect of various doses of a gonadotropin-releasing hormone analogue on induction of ovulation in anestrous mares. J. Anim. Sci. 72,178-183.

Murphy, B.D., Martinuk, S.D., 1991. Equine chorionic gonadotropin. Endocrine reviews 12(1):27-43.

Nakao, N., Ono, H., Yoshimura, T., 2008. Thyroid hormones and seasonal reproductive neuroendocrine interactions. Reproduction 136:1-8.

Nasser, L., Adams ,G.P., Bo, G.A., Mapletoft, R.J., 1993. Ovarian superstimulatory response relative to follicular wave emergence in heifers. Theriogenology 40,713–24.

Nasser, L.F., Sá Filho, M.F., Reis, E.L., Rezende, C.R., Mapletoft, R.J., Bó, G.A., Baruselli, P.S., 2011. Exogenous progesterone enhances ova and embryo quality following superstimulation of the first follicular wave in Nelore (*Bos indicus*) donors. Theriogenology 76 ,320–327.

Naylor, S.L., Chin, W.W., Goodman, H.M., Lalley, P.A., Grzeschik, K.H., Sakaguchi, A.Y., 1983. Chromosome assignment of genes encoding theα andB subunits of glycoprotein hormones in man and mouse. Somatic Cell Genetics 9,757-770.

Neta, A.V.C., Mol, J.P., Xavier, M.N., Paixão, T.A., Lage, A.P., Santos, R.L., 2010. Pathogenesis of bovine brucellosis. Vet. J. 184,146-155.

Newcomb, R., Christie, W.B., Rowson, L.E., 1978. Non-surgical recovery of bovine embryos. Vet. Rec. 102,414-417.

Nielsen K, Gall D, Jolley M, Leishman, G., Balsevicius, S., Smith, P., Nicoletti, P., Thomas F., 1996. A homogeneous fluorescence polarization assay for detection of antibody to *Brucella abortus*. J. Immunol. Method 195,161-168.

Nielsen, K., 2002. Diagnosis of brucellosis by serology. Vet. Microbiol. 90,447-459.

Niswender, K.D., Alvarenga, M.A., McCue, P.M., Hardy, Q.P., Squires, E.L., 2003. Superovulation in cycling mares using equine follicle stimulating hormone (eFSH). J. Equine Vet. Sci. 23,497-500.

Nowshari, M.A., Ali, S.A., 2005. Effect of season and gonadotropins on the superovulatory response in camel (Camelus dromedarius). Theriogenology 64, 1526–1535.

O'Malley, B.W., Strott, C.A., 1999. Steroid hormones: metabolism and mechanism of action. In: S.S.C Yen, R.B. Jaffe, R.L. Barbieri (Eds) *Reproductive Endocrinology. First edition.* Philadelphia, PA, USA, Elsevier Academic Press publications. p. 124.

Oliver, J.D., 2005. The viable but nonculturable state in bacteria. J. Microbiol. 43,93-100.

Olsen, S., Tatum, F., 2010. Bovine brucellosis. Vet. Clin. Food Anim. 26,15–27.

Olsen, S.C., 2010. Brucellosis in the United States: Role and significance of wildlife reservoirs. Vaccine 28S,F73–F76.

Olsen, S.C., Christie, R.J., Grainger, D.W., Stoffregen, W.S., 2006. Immunologic responses of bison to vaccination with Brucella abortus strain RB51: Comparison of parenteral to ballistic delivery via compressed pellets or photopolymerized hydrogels. Vaccine 24,1346-1353.

Olsen, S.C., Holland, S.D., 2003. Safety of revaccination of pregnant bison with Brucella abortus strain RB51. J. Wildl. Dis. 39,824-829.

Olsen, S.C., Jensen, A.E., Stoffregen, W.C., Palmer, M.V., 2003. Efficacy of calfhood vaccination with Brucella abortus strain RB51 in protecting bison against brucellosis. Res. Vet. Sci. 74,17-22.

Olsen, S.C., Kreeger, T.J., Schultz, W., 2002. Immune responses of bison to ballistic or hand vaccination with Brucella abortus strain RB51. J. Wildl. Dis. 38,738-745.

Osborn, J.C., Moor, R.M., 1983. The role of steroid signals in the maturation of mammalian oocytes. J. Steroid Biochem. 19,133–137.

Othen, L.S., Bellem, A.C., Gartley, C.J., Auckland, K., King, W.A., Liptraps, R.M., Goodrowel, K.L., 1999. Hormonal control of estrous cyclicity and attempted superovulation in wood bison (bison bison athabascae). Theriogenology 52,313-323.

Oussaid, B., Mariana, J.C., Poulin, N., Fontaine, J., Lonergan, P., Beckers, J.F., Cognie, Y., 1999. Reduction in the developmental competence of sheep oocytes by inhibition of LH pulses during the follicular phase with a GnRH antagonist. J. Reprod. Fertil. 117,71–77.

Palmer, M.V., Olsen, S.C., Gilsdorf, M.J., Philo, L.M., Clarke, P.R., Cheville, N.F., 1996. Abortion and placentitis in pregnant bison (Bison bison) induced by the vaccine candidate, *Brucella abortus* strain RB51. Am. J. Vet. Res. 57,1604-1607.

Palomino, J.M., McCorkell, R.B., Woodbury, M.R., Adams, G.P., 2014a. Ovarian synchronisation in wood bison (Bison bison athabascae) during the anovulatory season. Reprod. Fertil. Dev. 26,521-526.

Palomino, J.M., McCorkell, R.B., Woodbury, M.R., Cervantes, M.P., Adams, G.P., 2014b. Ovarian superstimulation and oocyte collection in wood bison (Bison bison athabascae) during the ovulatory season. Theriogenology 81,250-256.

Palomino, J.M., McCorkell, R.B., Woodbury, M.R., Cervantes, M.P., Adams, G.P., 2013. Superstimulatory response and oocyte collection in NorthAmerican bison during the non-breeding season. Anim. Reprod. Sci. 140,147-152.

Pappas, G., Panagopoulou, P., Christou, L., Akritidis, N., 2006. Biological weapons: *Brucella* as a biological weapon. Cell. Mol. Life Sci. 63:2229–2236.

Perry, G.A., 2014. Statistics of embryo collection and transfer in domestic farm bison. Newsletter of the International Embryo Transfer Society (IETS). 23,14-24.

Perry, G.A., Smith, M.F., Lucy, M.C., Green, J.A., Parks, T.E., MacNeil, M.D., Roberts, A.J., Geary, T.W., 2005. Relationship between follicle size at insemination and pregnancy success. PNAS 102, 5268–5273.

Perry, G.A., Smith, M.F., Roberts, A.J., MacNeil, M.D., Geary, T.W., 2007. Relationship between size of the ovulatory follicle and pregnancy success in beef heifers. J. Anim. Sci. 85, 684-689.

Pierce, J.G., Parsons, T.F., 1981. Glycoprotein hormones: structure and function. Annu. Rev. Biochem. 50,465-295.

Pieterse, M.C., Kappen, K.A., Kruip, T.A.M., Taverne, M.A.M., 1988. Aspiration of bovine oocytes during transvaginal ultrasound scanning of ovaries. Theriogenology 30,751–62.

Pieterse, M.C., Vos, P.L.A.M., Kruip, Th.A.M., Wurth, Y.A., 1991. Transvaginal ultrasound guided follicular aspiration of bovine oocytes. Theriogenology 35,857-862.

Pineda, M.H. 2003. Female reproductive system. In: M.H. Pineda, M.P. Dooley (Eds) *McDonald's Veterinary Endocrinology and Reproduction. Fifth Edition*. Iowa, USA, Iowa State Press. p. 283-340.

Poester, F.P., Nielsen, K., Samartino, L.E., Yu, W.L., 2010. Diagnosis of brucellosis. Open Vet. Sci. J. 4,46-60.

Poester, F.P., Samartino, L.E.,Santos, R.L., 2013. Pathogenesis and pathobiology of brucellosis in livestock. Rev. Sci. Tech. 32,105-115.

Pursley, J.R., Mee, M.O., Wiltbank, M.C., 1995. Synchronization of ovulation in dairy cows using PGF2, and GnRH. Theriogenology 44, 915-923.

Purwantara, B., Callesen, H., Greve, T., 1994. Characteristics of ovulations in superovulated cattle. Anim. Reprod. Sci. 37,1-5.

Radostits, O.M., Gay, C.C., Hinchcliff K.W., Constable P.D. 2007. Veterinary Medicine. *10th edition*. Philadelphia, PA, USA, Saunders, 2156 pp.

Rawlings, N., Evans, A.C.O., Chandolia, R.K., Bagu, E.T., 2008. Sexual maturation in the bull. Reprod. Dom. Anim. 43(s2),295-301.

Rawlings, N.C., Kennedy, S.W., Chang, C.H., Hill, J.R., Henricks, D.M., 1977. Onset of seasonal anestrus in the ewe. J. Anim. Sci. 44,791–797.

Reano, I., Carballo, D., Tribulo, A., Tribulo, P., Balla, E., Bo, G. A., 2009. Efecto de la adicion de eCG a los tratamientos superovulatorios con Folltropin-V en la produccion de embriones de donantes de embriones. In: Proceedings VIII Simposio Internacional de Reproduccion Animal – IRAC 2009. p. 54.

Ree, T.O., Colazo, M.G., Lamont, A.G.A., Kastelic, J.P., Dyck, M.K., Mapletoft, R.J., Ametaj, B.N., Ambrose, D.J. 2009. The effect of porcine luteinizing hormone in the synchronization of ovulation and corpus luteum development in non lactating cows. Theriogenology 72,120–128.

Reed, D.H., Frankham, R., 2003. Correlation between fitness and genetic diversity. Conserv. Biol. 17,230-237.

Reyna, J., Thomson, P.C., Evans, G., Maxwell, W.M.C., 2007. Synchrony of ovulation and follicular dynamics in merino ewes treated with GnRH in the breeding and non-breeding seasons. Reprod. Dom. Anim. 42, 410–417.

Richards, J.S., Russell, D.L., Robker, R.L., Dajee, M., Alliston, T.N., 1998. Molecular mechanisms of ovulation and luteinisation. Mol. Cell. Endocrinol. 145, 47–54

Riddell, K.P., Stringfellow, D.A., Gray, B.W., Riddell, M.G., Wright, J.C., Galik, P.K., 1993. Structural and viral association comparisons of bovine zonae pellucidae from follicular oocytes, day-7 embryos and day-7 degenerated ova. Theriogenology 40,1281-1291.

Rivera, F.A., Mendonça, L.G., Lopes, G., Santos, J.E., Perez, R.V., Amstalden, M., Correa-Calderon, A., Chebel, R.C., 2011. Reduced progesterone concentration during growth of the first follicular wave affects embryo quality but has no effect on embryo survival post transfer in lactating dairy cows. Reproduction 141,333-342.

Robertson, D.M., Foulds, L.M., Fry, R.C., Cummins, J.T., Clarke, I., 1991. Circulating half-lives of follicle-stimulating hormone and luteinizing hormone in pituitary extracts and isoform fractions of ovariectomized and intact ewes. Endocrinology 129, 1805-1813.

Robertson, E.G., 2015. Embryo collection and transfer. In: John Wiley & Sons, Inc. (Eds), *Bovine Reproduction*, Ed. Wiley Blackwell, p. 703-717.

Robinson, J.E., Radford, H.M., Karsch, F.J., 1985. Seasonal changes in pulsatile luteinizing hormone (LH) secretion in the ewe: Relationship of frequency of LH pulses to day length and response to estradiol negative feedback. Biol. Reprod. 33,324-334.

Robison, C.D., Davis, D.S., Templeton, J.W., Westhusin, M., Foxworth, W.B., Gilsdorf, M.J., Adams, L.J., 1998. Conservation of germ plasm from bison infected with *Brucella abortus*. J Wildl. Dis. 34, 582-589.

Roe, F.G., 1951. The North American buffalo. A critical study of the species in its wild state. University of Toronto press. Toronto. 957 pp.

Rosa, H.J.D., Bryant, M.J., 2003. Seasonality of reproduction in sheep. Small Rumin. Res. 48,155–171.

Rowe, R.F., Del Campo, M.R., Critser, M.S., Ginther, O.J., 1979. Embryo transfer in cattle: Nonsurgical collection techniques. Am. J. Vet. Res. 41,106-108.

Rowe, R.F., Del Campo, M.R., Eilts, C.L., French, L.R., Winch, R.P., Ginther, O.J., 1976. A single cannula technique for nonsurgical collection of ova from cattle. Theriogenology 6,471-483.

Rutley, B.D., Rajamahendran, R., 1995. Circannual reproductive function of female bison (*Bison bison*). In: Proceedings, Western section, American Society of Animal Science, Lethbridge, AB, Canada, 6-8 July. 46,242-245.

Ryan, J.P., Hunton, J.R., Maxwell, W.M.C., 1992. Time of ovulation in Merino ewes superovulated with PMSG and FSH-P. Reprod. Fertil. Dev. 4,91-79

Sanderson, E.W., Redford, K.H., Weber, B., Aune, K., Baldes, D., Berger, J., Carter, D., Curtin, C., Derr, J., Dobrott, S., Fearn, E., Fleener, C., Forrest, S., Gerlach, C., Gates, C.C., Gross, J.E., Gogan, P., Grassel, S., Hilty, J.A., Jensen, M., Kunkel, K., Lamers, D., List, R.,

Minkowski, K., Olson, T., Pague, C., Robertson, P.B., Stephenson, B. 2007. The ecological future of the North American bison: conceiving long-term, large-scale conservation of wildlife. Conserv. Biol. 22(2): 252–266.

Santos, J.E., Thatcher, W.W., Pool, L., Overton, M.W., 2001. Effect of human chorionic gonadotropin on luteal function and reproductive performance of high-producing lactating Holstein dairy cows. J. Anim. Sci. 79, 2881-2894.

Sartori, R., Fricke, P.M., Ferreira, J.C.P., Ginther, O.J., Wiltbank, M.C., 2001. Follicular deviation and acquisition of ovulatory capacity in bovine follicles. Biol. Reprod. 65, 1403-1409.

Sartori, R., Guardieiro, M.M., Barros, C.M., Bastos, M.R., Machado, G.M., Leme, L.O., Rumpf, R., 2009. Lack of improvement on embryo production by the replacement of the last two doses of pFSH by eCG in superstimulated Nellore heifers. Reprod. Fertil. Dev. 21,245–246.

Savio J.D., Keenan, L., Boland, M.P., Roche, J.F., 1988. Pattern of growth of dominant follicles during the estrus cycle of heifers. J. Reprod. Fertil. 83, 663-671.

Schmitt, E.J.P., Barros, C.M., Fields, P.A., Fields, M.J., Diaz, T., Kluge, J.M., Thatcher, W.W., 1996. A cellular and endocrine characterization of the original and induced corpus luteum after administration of a gonadotropin-releasing hormone agonist or human chorionic gonadotropin on day 5 of the estrous cycle. J. Anim. Sci. 74, 1915–1929.

Schumaker, B.A., Corso, B.A., Rhyan, J.C., Philo, L.M., Salman, M.D., Gardner, I.A., 2010. Evaluation of the fluorescence polarization assay for the detection of Brucella abortus antibodies in bison in a natural setting. Comp. Immunol. Microbiol. Infect. Dis. 33,e119-e125.

Seidel, G.E., 1981. Superovulation and embryo transfer in cattle. Science 211(4480),351-358.

Seleem, M.N., Boyle, S.M., Sriranganathan, N., 2010. Brucellosis: a re-emerging zoonosis. Vet. Microbiol. 140,392-398.

Senger, P.L., 2003. Pathways to Pregnancy and Parturition. Second edition. Current Conceptions, Inc., Washington State University, Pullman, WA, USA, pp. 368.

Shaw, J.H., Carter, T.S., 1989. Calving patterns among American bison. J. Wildl. Manage. 53,896-898.

Shury, T.K., Nishi, J.S., Elkin, B.T., Wobeser, G.A., 2015. Tuberculosis and brucellosis in wood bison (*Bison bison athabascae*) in Northern Canada: A renewed need to develop options for future management. J. Wildl. Dis. 51,1-12.

Singh, S.R., Hileman, S.M., Connors, J.M., McManus, C.J., Coolen, L.M., Lehman, M.N., Goodman, R.L., 2009. Estradiol negative feedback regulation by glutamatergic afferents to A15 dopaminergic neurons: variation with season. Endocrinology, 150,4663-4671.

Sirard, M.A., Richard, F., Blondin, P., Robert, C., 2006. Contribution of the oocyte to embryo quality. Theriogenology 65,126-136.

Solti, L., Crichtot, E.G., Loskutoff, N.M., Cseh, S., 2000. Economical and ecological importance of indigenous livestock and the application of assisted reproduction to their preservation. Theriogenology 53,149-162.

Soper, J.D., 1964. The mammals of Alberta. Hamly Press, Edmonton, Alberta, Canada. 402 pp.

Species at Risk Act, S.C., 2002. chapter 29. Published by the Minister of Justice. URL: http://laws-lois.justice.gc.ca/PDF/S-15.3.pdf (visited on 2015-07-14)

Speight, A., Popkin, R., Watts, A.G., Fink, G., 1981. Oestradiol-17β increases pituitary responsiveness by a mechanism that involves the release and the priming effect of luteinizing hormone releasing factor. J. Endocrinol. 88,301–308.

Stephenson, R.O., Gerlach, S.C., Guthrie, R.D., Harington, C.R., Mills, R.O., Hare, G., 2001. Wood bison in late Holocene Alaska and adjacent Canada: Paleontological, archaeological and historical records. BAR Int'l. Series 944,124-158.

Stevenson, J.S. 2007. Clinical reproductive physiology of the cow. In: R.S. Youngquist, W.R. Threlfall (Eds) *Current therapy in large animal theriogenology, Second edition*. St. Louis, Missouri, USA, Saunders. p. 259-270

Stevenson, J.S., Portaluppi, M.A., Tenhouse, D.E., Lloyd, A., Eborn, D.R., Kacuba, S., DeJarnette, J.M., 2007. Interventions after artificial insemination: Conception rates, pregnancy survival, and ovarian response to gonadotropin-releasing hormone, human chorionic gonadotropin, and progesterone. J. Dairy Sci. 90,331–340.

Stewart, F., Allen, W.R., 1979. The binding of FSH, LH and PMSG to equine gonadal tissues. J. Reprod. Fertil. 27,431–40.

Stewart, F., Allen, W.R., Moor, R.M., 1976. Pregnant mare serum gonadotrophin ratio of follicle-stimulating hormone and luteinizing hormone activities measured by radioreceptor assay. J. Endocrinol. 71,371–82.

Stringfellow, D.A., Givens, M.D. 2010. Manual of the International Embryo Transfer Society (IETS). 4th ed. Champaign, Illinois.

Stringfellow, D.A., Givens, M.D., 2000. Preventing disease transmission through the transfer of in-vivo-derived bovine embryos. Livest. Prod. Sci. 62,237-251.

Stringfellow, D.A., Panangala, V.S., Galik, P.A., 1988. Recovery and culture of ova from Brucella abortus infected cows. Theriogenology 29,1105-1112.

Stringfellow, D.A., Scanian, C.M., Brown, R.R., Meadows, G.B., Gray, B.W., Young-White, R.R., 1984. Culture of bovine embryos after in vitro exposure to *Brucella abortus*. Theriogenology 21,1005-1012.

Stringfellow, D.A., Wolfe, D.F., McGuire, J.A., Lauerman, L.H., Gray, B.W., Sparling, P.H., 1986. Effects of embryo-freezing and thawing techniques on the survivability of Brucella abortus. Theriogenology 26,553-559.

Stringfellow, D.A., Wright, J.C., 1989. A review of the epidemiologic aspects of embryo transfer from Brucella abortus-infected cows. Theriogenology 31,997-1006.

Strong, W.L., Gates, C.C., 2009. Wood bison population recovery and forage availability in northwestern Canada. J. Environ. Manage. 90,434-440.

Talmadge, K., Vamvakopoulos, N.C., Fiddes, J.C., 1984. Evolution of the genes for the /3 subunits of human chorionic gonadotropin and luteinizing hormone. Nature 307, 37-40.

Tessaro, S., 1986. The existing and potential importance of brucellosis and tuberculosis in Canadian wildlife: A review. Can. Vet. J. 27,119-124.

Tessaro, S.V., Forbes, L.B., Turcotte, C. 1990. A survey of brucellosis and tuberculosis in bison in and around Wood Buffalo National Park, Canada. Can. Vet. J. 31:174-180.

Tessaro, S.V., Gates, C.C., Forbes, L.B., 1993. The brucellosis and tuberculosis status of wood bison in the Mackenzie Bison Sanctuary, Northwest Territories, Canada. Can. J. Vet. Res. 57,231–235.

Thatcher, W.W., Drost, M., Savio, J.D., Macmillan, K.L., Entwistle, K,W., Schmitt, E.J., De la Sota, R.L., Morris, G.R. 1993. New clinical uses of GnRH and its analogues in cattle. Anim. Reprod. Sci. 33, 27-49.

The Western Producer. 2014. Thousands of cattle quarantined near Yellowstone. http://www.producer.com/wp-content/uploads/2014/12/20141218.pdf. Accessed August 2015.

Thiéry, J., Malpaux, B. 2003. Seasonal regulation of reproductive activity in sheep. Modulation of access of sex steroids to the brain. Ann. N.Y Acad. Sci. 1007, 169-175.

Thiéry, J.C., Chemineau, P., Hernandez, X., Migaud, M., Malpaux, B., 2002. Neuroendocrine interactions and seasonality. Dom. Anim. Endocrinol. 23,87–100.

Toosi, B.M., Tribulo, A., Lessard, C., Mastromonaco, G.F., McKorkell, R.B., Adams, G.P. 2013. Superovulation and embryo transfer in wood bison (Bison bison athabascae). Theriogenology 80, 542–551.

Tríbulo, A., Rogan, D., Tribulo, H., Tribulo, R., Alasino, R.V., Beltramo, D., Bó, G.A., 2011. Superstimulation of ovarian follicular development in beef cattle with a single intramuscular injection of Folltropin-V. Anim. Reprod. Sci., 129,7-13.

Tríbulo, A., Rogan, D., Tríbulo, H., Tríbulo, R., Mapletoft, R.J., Bó, G.A., 2012. Superovulation of beef cattle with a split-single intramuscular administration of Folltropin-V in two concentrations of hyaluronan. Theriogenology, 77,1679-1685.

Van Soom, A., Wrathall, A.E., Herrler, A., Nauwynck, H.J., 2009. Is the zona pellucida an efficient barrier to viral infection?. Reprod. Fertil. Dev. 22,21-31.

van Zyll de Jong, C.G., 1986. A systematic study of recent bison, with particular consideration of the wood bison (*Bison bison athabascae*). Nat. Sci. 6,1–69.

van Zyll de Jong, C.G., Gates, C., Reynolds, H., Olson, W., 1995. Phenotypic variation in remnant populations of North American bison. J. Mammalogy 76,391-405.

Vasconcelos, J.L.M., Sartori, R., Oliveira, H.N., Guenther, J.G., Wiltbank, M.C. 2001. Reduction in size of the ovulatory follicle reduces subsequent luteal size and pregnancy rate. Theriogenology 56,307-314.

Vervaecke, H., Roden, C., de Vries, H., 2005. Dominance, fatness and fitness in female American bison, Bison bison. Anim. Behav. 70,763-770.

Vervaecke, H., Schwarzenberger, F. 2006. Endocrine and behavioral observations during transition of non-breeding into breeding season in female American bison (*Bison bison*). Theriogenology 66:1107-1114.

Vos, P.L.A.M., Bevers, M.M., Willemse, A.H., Dieleman, S.J., 1994. Effects of suppression by a progesterone releasing intravaginal device and subsequent induction by GnRH of the preovulatory LH surge on follicular function in PMSG/PG treated heifers. J. Reprod. Fertil. 101,43–49.

Walker, S.K., Smith, D.H., Seamark, R.F., 1986. Timing of multiple ovulations in the ewe after treatment with FSH or PMSG with and without GnRH. J. Reprod. Fert. 77,135-142.

Wani, G.M., Buchoo, B.A., Wani, N.A., 1997. Use of human gonadotrophin in superovulation of Southdown sheep. Small Rumin. Res. 25,93-94.

Wassarman, P.M., 1988. Zona pellucida glycoproteins. Annu. Rev. Biochem. 57,415-442.

Wildt, D.E. 1992. Genetic resource banks for conserving wildlife species: justification, examples and becoming organized on a global basis. Anim. Reprod. Sci. 28, 247-257.

Wilson, G.A., Nishi, J.S., Elkin, B.T., Strobeck, C., 2005. Effects of a recent founding event and intrinsic population dynamics on genetic diversity in an ungulate population. Conservation Genetics 6,905-916.

Wubishet, A., Graves, C.N., Spahr, S.L., Kesler, D. J., Favero, R. J., 1986. Effects of GnRH treatment on superovulatory responses of dairy cows. Theriogenology, 25,423-427.

Xavier, M.N., Paixão, T.A., Poester, F.P., Lage, A.P., Santos, R.L., 2009. Pathological, immunohistochemical and bacteriological study of tissues and milk of cows and fetuses experimentally infected with Brucella abortus. J. Comp. Pathol. 140,149-157.

Yadav, M.C., Walton, J.S., Leslie, K.E., 1986. Timing of the onset and duration of ovulation in superovulated beef heifers. Theriogenology 26,509-521.

Yang, X., Skyberg, J.A., Cao, L., Clapp, B., Thornburg, T., Pascual, D.W., 2013. Progress in Brucella vaccine development. Front. Biol. 8,60-77.

Yavas, Y., Johnson, W.H., Walton, J.S., 1999. Modification of follicular dynamics by exogenous FSH and progesterone, and the induction of ovulation using hCG in postpartum beef cows. Theriogenology 52,949‑963.

Yen, S.S.C., 1999. Neuroendocrinology of reproduction. In: S.S.C. Yen, R.B. Jaffe, R.L. Barbieri (Eds) *Reproductive Endocrinology. Physiology, Pathophysiology, and Clinical Management. Fourth Edition*. Philadelphia, PA, USA, W.B. Saunders Company. p. 30-80.

Young, E. J., 1995. An overview of human brucellosis. Clin. Infect. Dis. 21,283-289.